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MECHANISMS IN ETHANOL MODULATION OF GABA RELEASE ONTO DOPAMINERGIC NEURONS OF THE VENTRAL TEGMENTAL AREA

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by

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MECHANISMS IN ETHANOL MODULATION OF GABA RELEASE ONTO DOPAMINERGIC NEURONS OF THE VENTRAL TEGMENTAL AREA

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Activation of ventral tegmental area (VTA) dopaminergic (DA) neurons by ethanol has been implicated in the rewarding and reinforcing actions of ethanol. GABAergic transmission is thought to play an important role in regulating the activity of DA neurons. While at most central synapses ethanol generally increases inhibitory synaptic transmission, no studies have explored the effect of acute ethanol on GABAergic transmission in the VTA. Here we investigated how ethanol modulates GABAergic transmission in the VTA in relation to the overall action of ethanol on VTA-DA neuron activity. We demonstrated that ethanol dose-dependently enhances action potential-dependent and -independent GABA release onto VTA-DA neurons. Utilizing whole-cell voltage clamp recording techniques, ethanol increased both spontaneous and miniature inhibitory postsynaptic current (s/mIPSC) frequency while having minimal effect on s/mIPSC amplitude. The ethanol enhancement in GABA release was independent of $GABA_B$ auto-receptor inhibition of release. Intra-terminal calcium

levels regulate neurotransmitter release, thus we investigated how modulation of calcium levels would affect the ethanol-enhancement in GABA release. Ethanol enhanced mIPSC frequency in the presence of the voltage-gated calcium channel blockers, cadmium chloride and nicardipine. However, blockade of intracellular calcium stores with 2-APB and cyclopiazonic acid eliminated the ethanol-enhancement of mIPSC frequency. Intracellular calcium stores are regulated via G_q protein-coupled receptors such as the 5-HT_{2C} receptor. 5-HT_{2C} receptor activation robustly enhanced mIPSC frequency whereas blockade inhibited the ethanol-enhancement in mIPSC frequency. These observations suggest that increased calcium release from intracellular stores via 5-HT_{2C} receptor activation is involved in the ethanol-enhancement of GABA release onto VTA-DA neurons. Utilizing cell-attached current-clamp recordings, we demonstrated that the ethanol-enhancement of VTA-DA neuron activity is modulated by the concurrent enhancement in GABA release. Blockade and activation of $GABA_A$ receptors enhanced and reversed, respectively, the stimulatory effect of ethanol on VTA-DA neurons. Mu-opioid receptors (MORs) on GABAergic interneurons have been demonstrated to modulate both basal and ethanol-enhanced VTA-DA activity *in vivo*, though we failed to demonstrate such an effect *in vitro*. Overall, the results of this study suggest that the 5-HT_{2C} receptor and intra-terminal calcium-dependent ethanol enhancement in GABA release acts to regulate the overall stimulatory effect of ethanol on VTA-DA activity.

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Abbreviations

3n	oculomotor nerve
5-HT	5-hydroxytryptamine, serotonin
aCSF	artificial cerebrospinal fluid
AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazole proprionate
AMY	amygdala
ANOVA	analysis of variance
BLA	basolateral amygdala
CeA	central nucleus of amygdala
CIE	chronic intermittent ethanol
CPA	cyclopiazonic acid
DA	dopaminergic
DOR	delta opioid receptor
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GPCR	G protein-coupled receptor
I_h	hyperpolarization-activated cationic current
IP_3	inositol-1,4,5- triphosphate
IPSC	inhibitory postsynaptic current
KOR	kappa opioid receptor
mIPSC	miniature IPSC
ml	medial lemniscus
MOR	mu opioid receptor
mPFC	medial prefrontal cortex
MT	medial terminal nucleus of the accessory optic tract
NAc	nucleus accumbens
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NMDA	N-methyl D-aspartate
PBP	parabrachial pigmented area
PFC	prefrontal cortex
PFR	parafasciculus retroflexus area
PLC	phospholipase C
PN	paranigral nucleus
SERCA	sarco/endoplasmic reticulum calcium ATPase
sIPSC	spontaneous IPSC
SNc	substantia nigra compacta
SNr	substantia nigra reticulata
TH	tyrosine hydroxylase
TTX	tetrodotoxin
VGCC	voltage-gated calcium channel
VTA	ventral tegmental area
VTT	ventral tegmental tail

Chapter 1: Introduction

1-1. Alcohol dependence

1-1-1. Definition

'Addiction' as a disease concept is recognized by the biomedical community and is defined by a specific set of criteria. According to the *Diagnostic and Statistical Manual of Mental Disorders*, the American Psychiatric Association (DSM-IV; APA 1994) for clinicians uses the term 'substance dependence' instead of 'addiction' in referring to a set of seven symptoms indicative of a persistent and uncontrolled use of a substance despite serious negative consequences. The criteria for differentiating substance dependence versus substance abuse involves the presence of three or more of the listed seven cardinal symptoms manifesting themselves in any one twelve-month period. These symptoms include (1) tolerance, (2) withdrawal, (3) prolonged or excessive use, (4) persistent desire to continue use, (5) excessive time allotted to drug seeking and use, (6) negative impacts on social, occupational and recreational activities resulting from use and (7) continued drug use despite the user's knowledge of negative drug-induced problems. These criteria used to diagnose substance dependence apply to all drug types regardless of pharmacological action. However, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines 'alcoholism' or 'alcohol dependence' to the lay person as a disease that includes the following four symptoms: craving, loss of control, physical dependence, and tolerance. However, dependence does not

come about solely due to excessive use. In fact, it is well established that drug dependence consists of two major components: (1) a genetic predisposition whereby 40-60% of the vulnerability to dependence can be attributed to genetic factors (Goldman et al., 2005; Hiroi and Agatsuma, 2005) and (2) the abuse component whereby excessive drug use results in synaptic and non-synaptic dysfunctions that over a long period of time result in compensatory changes that manifests itself into the addictive state.

1-1-2. Economic and societal impact

According to a polled study conducted during 1990-1992, 91.5% of American adults reported experience with alcohol, with 14% of those being dependent (Anthony et al., 1994). These data support the notion that alcohol abuse and dependence is particularly pervasive in this country. According to the NIAAA, alcohol abuse has a substantial economic cost on the United States, with an estimated societal cost of $185 billion in 1998. This estimate includes the costs associated with health care expenditures, productivity impacts, motor vehicle crashes, crime, social welfare administration and other factors. In 2004, 40% of all traffic crash fatalities were alcohol related (Alcohol Epidemiologic Data System, NIAAA, 2006). Obviously, alcohol abuse and dependence have major impacts on our society and therefore the necessary resources should be made available in order to further understand the disease to find better treatments.

1-1-3. Current treatment

There is no cure for alcoholism; however, there are several available medications that have displayed limited effectiveness in some people in promoting abstinence from alcohol consumption. These drugs include: naltrexone, disulfiram, acamprosate and topiramate (Brunton et al., 2006). In 1994, the Food and Drug Administration approved the use of naltrexone, a non-selective opioid receptor antagonist, for therapeutic use in alcohol dependence (Franklin, 1995). The therapeutic utility of naltrexone is believed to be mediated through a dampening of the excitatory effects of alcohol on the mesolimbic system. However, the precise mechanism of naltrexone in mediating this effect is still unclear. Dilsufiram is used as a deterrent to alcohol consumption. When given alone disulfiram is relatively innocuous. However, due to its action as an inhibitor of aldehyde dehydrogenase, adjunctive alcohol consumption results in a buildup of acetaldehyde thus causing the patient to become very ill. Other drugs which show some effectiveness in treatment, although the mechanisms are even less clear, include the gamma-aminobutyric acid (GABA) analogue, acamprosate, and topiramate, which is also used to treat seizure disorders (Brunton et al., 2006).

1-2. Ventral Tegmental Area

1-2-1. Anatomy and connectivity

A primary system involved in drug dependence is the mesocorticolimbic dopamine pathway, originating in the ventral tegmental area (VTA). The VTA

was first described in the opossum brain, although it was originally referred to as the ventral tegmental nucleus before adopting its more common nomenclature (Tsai, 1925). The VTA is also synonymous with the A10 nucleus, a term used to classify areas containing dopaminergic (DA) cell bodies along with two other DA nuclei in the ventral midbrain, the A8 and A9, referring to the retrorubral field (RRF) and substantia nigra pars compacta (SNc), respectively (Ungerstedt, 1971). In a horizontal section, the VTA is situated posterior to the lateral hypothalamic area (LHA), medial to the SNc and medial terminal nucleus of the accessory optic tract (MT) and anterior to the oculomotor nerve (3n) and medial lemniscus (ml). Although not necessarily consistent across species, the VTA can further be subdivided into four distinct regions: paranigral nucleus (PN), parabrachial pigmented area (PBP), parafasciculus retroflexus area (PFR) and the ventral tegmental tail (VTT) (Ikemoto, 2007). The PN and PBP, located respectively in the posterior ventromedial and anterior ventrolateral areas of the VTA contain a high density of DA cell bodies, whereas the PFR and VTT contain a low density of DA cell bodies.

The VTA consists primarily of DA and non-DA cell bodies which project to and receive inputs from a variety of mesocorticolimbic structures. DA cell bodies from the posteromedial regions of the VTA, including the PN, project to the ventromedial striatum, consisting of the medial nucleus accumbens (NAc) shell and the medial olfactory tubercle, whereas DA cell bodies in the lateral VTA, including the PBP, send projections to the lateral tubercle, lateral NAc shell,

NAc core and basolateral amygdala (BLA) (Ikemoto, 2007). VTA-DA cell bodies also send projections to the medial prefrontal cortex (mPFC) (Berger et al., 1976), lateral septum (Lindvall, 1975), amygdala (Ungerstedt, 1971; Albanese and Minciacchi, 1983) and hippocampus (Swanson, 1982). VTA-DA neurons projecting to the mPFC and BLA originate primarily from the PN and the ventromedial PBP (Ford et al., 2006; Lammel et al., 2008).

The VTA also receives reciprocal excitatory and inhibitory innervations from multiple brain regions. Glutamatergic inputs to the VTA arise from the mPFC, subthalamic nucleus, and the pedunculopontine nucleus (Christie et al., 1985; Groenewegen and Berendse, 1990; Sesack and Pickel, 1992; Charara et al., 1996). Glutamatergic inputs from the mPFC synapse onto both DA and GABAergic neurons in the VTA (Carr and Sesack, 2000b). A recent study utilizing retrograde tracer labeling demonstrated that nearly all brain structures projecting to the VTA send glutamatergic afferents (Geisler et al., 2007), thus supporting the idea that the VTA integrates excitatory information from a variety of areas. GABAergic neurons from the NAc and ventral pallidum project to VTA (Walaas and Fonnum, 1980), and VTA-GABAergic neurons project to both the mPFC (Carr and Sesack, 2000a) and the NAc (Van Bockstaele and Pickel, 1995). Additionally, local VTA GABAergic neurons (interneurons) project onto neighboring VTA-DA cells and are thought regulate the activity of these DA cells via tonic inhibition (Johnson and North, 1992a). Additional brain regions which

innervate the VTA include serotonergic afferents from the midbrain raphe nuclei that innervate both DA and GABAergic neurons (Herve et al., 1987).

1-2-2. Cell types and electrophysiological properties

Neurons in the VTA are classified as primary, secondary and tertiary (Grace and Onn, 1989; Johnson and North, 1992b; Cameron et al., 1997; Neuhoff et al., 2002). Primary neurons are considered dopaminergic and can be identified by the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme involved in the biosynthesis of dopamine. Primary and tertiary neurons both exhibit a slowly activating inward current in response to a hyperpolarizing step that is mediated via cyclic nucleotide-regulated cation channels (HCN, I_h). Additionally, both cell types display pacemaker firing (1-5 Hz) *in vitro* and long-duration action potentials. Secondary cells contain GABA and do not possess an I_h. They have shorter action potentials and exhibit rapid firing (>8 Hz), although one group has identified a subset of TH(-) VTA cells that exhibit very slow firing (<1 Hz) (Korotkova et al., 2003).

Primary and tertiary neurons are differentiated by their responses to mu-opioid receptor (MOR) activation. MOR agonists inhibit tertiary neurons and less than one-third of these cells are TH(+) (Cameron et al., 1997). Alternatively, MORs agonists either have no effect or disinhibit primary cells via inhibition of local GABAergic interneurons (Johnson and North, 1992a; Margolis et al., 2003). It should be noted however, that disinhibition of principal cells is not reliably demonstrated *in vitro*, possibly due to the loss of VTA circuitry during slice

preparation. Margolis et al. (2003) observed a disinhibitory effect by the MOR agonist, DAMGO, in less than half of the principle cells studied. Additionally, in the Johnson and North (1992a) study, a high extracellular K^+ concentration was required to potentiate GABA tone in order to see a disinhibitory effect of MOR activation. kappa-opioid receptor (KOR) activation inhibits a subset of principal and tertiary cells but not secondary cells (Margolis et al., 2003). Secondary cells are also inhibited by MOR agonists (Lacey et al., 1989; Johnson and North, 1992a).

Based on the aforementioned studies, it appears that the original classification of two distinct neuron subtypes in the VTA, DA and GABAergic, is a gross oversimplification of the neuronal population in this brain region. Indeed, some VTA cells express both TH and glutamic acid decarboxylase (GAD), a marker for GABAergic cells. One study showed that 27% of VTA cells express both (Klink et al., 2001) and in another study about 13% of VTA cells examined expressed both markers (Korotkova et al., 2003). Additionally, one study suggests that glutamatergic neurons are also present within the VTA (Yamaguchi et al., 2007). One study in particular claims that there is no reliable physiological marker selective for DA neurons in the VTA, including: cell morphology, I_h size, spontaneous firing rate, input resistance, dopamine D_2 receptor agonist inhibition and action potential duration (Margolis et al., 2006). However, the authors do concede that I_h(-) neurons are reliably non-DA, so using this criteria for distinguishing between DA and non-DA neurons does have some merit.

Additionally, even immunohistochemical classification of DA neurons is not without its problems. In mice, as the duration of the whole-cell recording increases, the effectiveness of post-hoc classification of recorded neurons diminishes (Zhang et al., 2008). Furthermore, the authors contend that I_h is indeed sufficient to differentiate between DA and non-DA cells in the VTA.

1-2-3. Neural substrates of addiction

1-2-3-1. Mesocorticolimbic system

DA neurons of the VTA constitute the central locus of the mesocorticolimbic dopamine system and send projections to the NAc, PFC, BLA and a variety of other corticolimbic structures (Albanese and Minciacchi, 1983; Oades and Halliday, 1987). Most drugs of abuse pharmacologically activate VTA-DA neurons via a variety of mechanisms culminating in aberrant release of DA onto these targets. Such pathological activation of the mesolimbic pathway is considered a primary step in the development and expression of drug dependence and addiction.

1-2-3-2. Dopamine and reward/reinforcement

An issue that has been hotly debated during the past few decades is the precise role of dopamine in both reward and reinforcement (Wise, 2004). Since dopamine levels rise during consumption of reward, dopamine is perceived as the ‘pleasure signal’ which mediates the hedonic qualities of both natural rewards (food, drink, sex) and drugs of abuse. This theory is termed the ‘anhedonia hypothesis’ because pharmacological blockade of the mesolimbic dopamine

system blocks the hedonic properties of food rewards (Wise et al., 1978). Therefore, dopamine was initially believed to be required for a stimulus to be perceived as 'good'. However, this is not necessarily true. Several studies support the idea that a hedonic state can be achieved even in the absence of dopamine (Berridge et al., 1989; Treit and Berridge, 1990; Berridge and Robinson, 1998; Cannon and Palmiter, 2003; Robinson et al., 2005). Developed from these studies was the 'incentive salience' hypothesis which states that dopamine is not required for 'liking' a reward, but is required for 'wanting' a reward (Berridge and Robinson, 1998). This hypothesis claims that dopamine is required for converting an otherwise neutral stimulus associated with a reward into an attractive or desirable quality that motivates the animal to obtain the reward. This theory transforms the role of dopamine from the early view of a passive consequence of reward consumption to a more active role in regulating the learning associated with motivational aspects pertained to seeking and obtaining a reward.

Evidence also suggests a role for dopamine in 'reinforcement', which is a term used to describe the creation of a strong association between a rewarding stimulus and the behavior that led to obtaining that rewarding stimulus. Support for the role of dopamine in reinforcement comes from numerous *in vivo* studies. Animals will normally learn to lever-press to obtain a reward, but not under conditions of dopamine blockade (Wise and Schwartz, 1981). Additionally, under dopamine blockade, animals fail to exhibit preference for environments

(conditioned place preference) associated with the receipt of a reward (Spyraki et al., 1982; Spyraki et al., 1983). These studies suggest that dopamine is required in order to learn that a particular behavior will result in a reward.

In a landmark series of behavioral studies with primates, phasic activation of dopamine neurons was shown to function as a reward predictor (Schultz et al., 1993; Mirenowicz and Schultz, 1994; Schultz et al., 1997). In these studies, when a reward (sip of fruit juice) was administered in the absence of a preceding conditioned stimulus, dopamine neurons produced a phasic increase in activity upon administration of a reward. However, after learning, the increase in dopamine neuron activity occurred at the presentation of the conditioned stimulus and no phasic response occurred during administration of the reward. Conversely, when the expected reward was withheld, there was a phasic decrease below the tonic activity of dopamine neurons at the time the reward should have been delivered. Thus, changes in dopamine neuron firing patterns are positively correlated with an unexpected or better than expected reward, and negatively correlated with a reward that is less than expected. Therefore, DA neuron activity may be important for processing predictive cues and/or influencing reward-maximizing behaviors, rather than a direct representation of a hedonic state as a consequence of the rewarding stimuli.

1-2-4. Ethanol and the mesolimbic system

1-2-4-1. Ethanol enhances dopamine release

It is well established that dopamine transmission within the mesocorticolimbic system is a critical component in the regulation of alcohol drinking behaviors. Systemic administration of dopamine agonists and antagonists reduced alcohol drinking in rats (Pfeffer and Samson, 1986; Pfeffer and Samson, 1988). Local microinjection of dopamine antagonists and agonists into the NAc terminated and prolonged ethanol drinking, respectively (Samson et al., 1992). Additionally, D_1 and D_2 receptor antagonism reduced ethanol seeking behavior in rats (Czachowski et al., 2001; Liu and Weiss, 2002), and genetic deletion of these receptors in mice attenuates alcohol-seeking and consumption (El-Ghundi et al., 1998; Phillips et al., 1998). *In vivo* microdialysis studies have demonstrated an increase in dopamine levels in the NAc in P and Wistar rats prior to and during ethanol self-administration (Weiss et al., 1993; Weiss et al., 1996; Gonzales and Weiss, 1998; Melendez et al., 2002).

Several recent studies have demonstrated that the ethanol-induced increase in dopamine release to the NAc is subregion dependent. Intravenous ethanol administration produced a greater increase in dopamine levels in the NAc shell as compared to the core, with the core only seeing a significant increase in dopamine at the highest ethanol concentration tested (Howard et al., 2008). Neither the shell nor core exhibited a significant increase in dopamine release during operant ethanol consumption, however, the core-shell border did exhibit an increase in dopamine release under this paradigm (Howard et al., 2009). Since VTA subregions project topographically to the NAc, these studies

suggest that the projections from the medial VTA may be more important in the rewarding or reinforcing properties of ethanol.

1-2-4-2. Direct and indirect effects on VTA-DA cell excitation

Acute ethanol has been shown to increase the firing rate of DA neurons in the VTA both *in vivo* (Gessa et al., 1985) and *in vitro* (Brodie et al., 1990). The latter study demonstrated a dose-dependent increase in spontaneous firing rate that was assumed to be largely attributable to a direct postsynaptic effect since the recordings were conducted in low calcium, high magnesium conditions to block neurotransmitter release. Additionally, the firing rate of acutely dissociated VTA-DA neurons was also enhanced by ethanol demonstrating that ethanol still enhances DA neuron excitability even in the absence of synaptic input (Brodie et al., 1999b). The ethanol enhancement in firing frequency may be due to a decrease in the after-hyperpolarization (AHP) amplitude and/or an enhancement in the I_h, both important modulators in determining the inter-spike interval (Brodie and Appel, 1998). These authors later demonstrated that, in Fisher-344 rats, block of I_h with external cesium or ZD7288 did not modulate ethanol-enhancement of VTA-DA firing rate (Appel et al., 2003). However, in C57BL/6J mice, ethanol enhances I_h and may contribute to the excitatory effects on VTA-DA firing (Okamoto et al., 2006), thus suggesting that the involvement of I_h in ethanol-enhancement of DA excitability differs across species. This phenomenon was examined again by Brodie and colleagues and they did not observe an effect of I_h block on the peak excitatory effect of ethanol on firing rate

in C57 and DBA mice; however, in the presence of ZD7288 ethanol produces an inhibitory effect on firing rate immediately following an excitatory response (McDaid et al., 2008). Additionally, block of SK-type of calcium-dependent K^+ current did not affect VTA-DA neuron firing rate, but did potentiate the ethanol-enhancement (Brodie et al., 1999a). These results suggest that a reduction in the SK current, which contributes to the AHP, does not contribute to the excitatory effects of ethanol on DA firing but rather the amount of SK current determines the ethanol sensitivity of these neurons. Other ethanol targets that may contribute to the enhancement in firing include a quinidine-sensitive delayed rectifier K^+ current (Appel et al., 2003) and the voltage-gated K^+ current, I_M (Koyama et al., 2007).

Upon ingestion, ethanol is metabolized to acetaldehyde (ACD) in the periphery by gastric and liver alcohol dehydrogenase (ADH) and in the CNS via a brain catalase/H_2O_2 system. One group contends that the excitatory effect of ethanol on the mesolimbic system is not a direct effect of ethanol *per se*, but rather due to an interaction with ACD (Melis et al., 2007). The authors demonstrated that ethanol-induced conditioned place preference is prevented with pre-treatment with 4-methyl-pryazole (4-MP), which blocks peripheral metabolism of ethanol to ACD. The authors also observed that the ethanol-induced increase in DA release in the NAc is blocked with 4-MP pre-treatment and local administration of ACD to the VTA increases DA release as measured via microdialysis. Additionally, application of ACD increased the firing rate of

VTA-DA neurons *in vitro*; moreover, co-application of 3-aminotriazole, a catalase inhibitor, blocked the ethanol-induced increase in DA firing rate. The authors further contend that the excitatory effects of ACD on firing rate are mediated via activation of I_h and inhibition of A-type K^+ channels.

1-2-5. Neurotransmitter receptors

1-2-5-1. GABA receptors

GABA is the main inhibitory neurotransmitter in the adult central nervous system. There are three GABA receptors: $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels permeable to monovalent anions, such as Cl^- and HCO_3^-, and mediate fast synaptic inhibition. The receptors are part of a superfamily of cys-loop ligand-gated ion channels which includes nicotinic acetylcholine, glycine and 5-HT_3 receptors. The $GABA_A$ receptor contains five subunits, with some containing several isoforms (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-3}) (Barnard et al., 1998). The most common receptor subtype contains two α, two β and one γ subunits in the following arrangements: $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$ (Benke et al., 1994; Sigel et al., 2006). Under typical physiological conditions, activation of $GABA_A$ receptors results in a flux of Cl^- ions into the cell, hyperpolarizing and thus decreasing the excitability of the cell. However, conditions do arise when $GABA_A$ activation can depolarize the membrane. $GABA_A$ receptors are located at synaptic and extrasynaptic locations. γ_2-containing receptors are expressed primarily at the synapse (Somogyi et al., 1996; Essrich et al., 1998) and mediate fast or ‘phasic’ inhibition.

δ-containing receptors, which associate primarily with $\alpha_{4/6}$ and $\beta_{2/3}$ subunits are expressed exclusively at extrasynaptic locations (Nusser et al., 1998) and mediate persistent or 'tonic' inhibition as a result of GABA-spillover from the synapse (Kaneda et al., 1995).

$GABA_A$ receptors are present on VTA-DA neurons and GABA release tonically inhibits these neurons (Yim and Mogenson, 1980; Johnson and North, 1992a; Johnson and North, 1992b; Westerink et al., 1996). In the SNc and VTA, DA neurons contain the α_{2-4}, $\beta_{1/3}$ and γ_2 receptor subunits (Okada et al., 2004), thus suggesting that $GABA_A$ receptors in the midbrain are localized primarily to synaptic sites mediating phasic inhibition. Interestingly, some reports suggest the majority of $GABA_A$ receptors are expressed on GABAergic cells and activation of these receptors results in disinhibition, rather than inhibition, of DA neurons in the VTA (Kalivas et al., 1990; Churchill et al., 1992; Xi and Stein, 1998; Laviolette and van der Kooy, 2001; Doherty and Gratton, 2007).

Metabotropic $GABA_B$ receptors are heterodimers composed of two subunits (GB1 and GB2) and belong to the class III group of G protein-coupled receptors (GPCRs). $GABA_B$ receptors are located at presynaptic and postsynaptic sites (Misgeld et al., 1995). Activation of postsynaptic $GABA_B$ receptors decreases excitability via opening of a G_i protein-coupled inward rectifying K^+ channel (GIRK). Activation of presynaptic $GABA_B$ receptors inhibits neurotransmitter release via inhibition of voltage-gated Ca^{2+} channels (VGCCs).

$GABA_B$ receptors are located at pre- and postsynaptic sites in the VTA with presynaptic $GABA_B$ receptor activation resulting in a decrease in GABA release (Melis et al., 2002). However, some argue that the majority of $GABA_B$ receptors are at postsynaptic sites on DA cells (Xi and Stein, 1998) and activation of these receptors with baclofen decreases DA output to the NAc (Westerink et al., 1996; Amantea and Bowery, 2004). GIRK-linked $GABA_B$ receptors are expressed on both GABAergic and DA cells in the VTA (Cruz et al., 2004). In that study, the authors demonstrated that GABAergic and DA cells exhibit cell-specific expression of GIRK subunits which have different coupling efficacies (EC_{50}) for baclofen. As a result, low baclofen doses inhibit GABAergic neurons (low EC_{50}) and high baclofen doses inhibit DA neurons. Thus due to differential expression of $GABA_A$ and $GABA_B$ receptors within the VTA there exists the potential for differential involvement of these receptors in reward and reinforcement.

1-2-5-1-1. Ethanol and $GABA_A$ receptors

Ethanol intoxication and $GABA_A$ receptor activation produce sedative and anxiolytic states which led many to believe that ethanol exerted its action on the CNS via direct interactions with postsynaptic $GABA_A$ receptors. Supporting evidence for this theory has emerged over the past few decades, although with mixed results (Mihic, 1999; Criswell and Breese, 2005). Discrepancies between studies in the effects of ethanol on $GABA_A$ receptor function can be due to a variety of reasons including, but not limited to: brain regions studied;

methodological factors; *in vitro* preparation used; electrophysiological recording configuration; and concentration of ethanol used (Weiner and Valenzuela, 2006).

A major issue at the center of debate regarding ethanol sensitivity of postsynaptic $GABA_A$ receptors concerns the exact subunit composition of the channel. Synaptic localization of $GABA_A$ receptors is subunit-dependent; γ-containing receptors are localized primarily to the synapse and mediate phasic inhibition whereas δ-containing receptors are primarily extrasynaptic and mediate tonic inhibition. Early studies using electrophysiological methods initially demonstrated a likely role for ethanol in potentiating γ-containing $GABA_A$ receptor function. In studies using oocytes (Wafford et al., 1991) and mouse L(tk^-) cells (Harris et al., 1995), ethanol was shown to enhance $GABA_A$ receptor function, but only in those receptors containing γ_{2L} subunits. Additionally, ethanol (1-50 mM) enhanced $GABA_A$ mediated currents in cultured mouse hippocampal and rat cortical neurons (Reynolds et al., 1992). However, many later studies failed to demonstrate any $GABA_A$ receptor sensitivity to ethanol at physiological concentrations (Criswell and Breese, 2005), thus fueling the debate regarding the role of postsynaptic $GABA_A$ receptors in mediating the physiological effects of ethanol. It has been shown that δ-containing $GABA_A$ receptors appear to be sensitive to low ethanol concentrations (<30 mM) (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). Additionally, Wallner et al (2003) demonstrated that γ-containing recombinant receptors were relatively insensitive to ethanol at concentrations below 100 mM. Then again, several groups have been unable to

replicate the sensitivity of δ-containing $GABA_A$ receptors to low ethanol concentrations (Borghese et al., 2006; Yamashita et al., 2006), thus further muddling the literature concerning the direct postsynaptic GABAmimetic action of ethanol (Santhakumar et al., 2007).

1-2-5-1-2. Ethanol and GABA release

Although there are discrepancies regarding the postsynaptic effects of ethanol, there is a general agreement that acutely administered ethanol facilitates presynaptic GABA release in most, but not all brain regions (Siggins et al., 2005; Weiner and Valenzuela, 2006). Surprisingly, the notion that ethanol could modulate GABA release went largely unexplored after early reports in the 1980s demonstrated an ethanol-enhancement of postsynaptic $GABA_A$ receptor function. Nearly 20 years would pass before the emergence of clear evidence of a potentiating effect of ethanol on presynaptic GABA release. Several electrophysiological methods can be used to detect changes in neurotransmitter release. Two methods commonly used for *in vitro* slice preparations include the analysis of changes in quantal, miniature (action potential independent; tetrodotoxin-insensitive) and spontaneous (action potential-dependent; tetrodotoxin-sensitive) inhibitory postsynaptic currents (m/sIPSCs). A change in the frequency of m/sIPSCs reflects a change in the number of release events; a change in the amplitude of m/sIPSCs represents a change in postsynaptic receptor sensitivity. Another method to study neurotransmitter release is by analyzing the change in the ratio of paired evoked synaptic potentials (paired-

pulse ratio; PPR). A common non-electrophysiological method involves the direct measurement of neurotransmitter levels via microdialysis *in vivo*.

1-2-5-1-2-1. Hippocampus

The hippocampus is an important component of the mesolimbic system and is critically involved in learning and memory. Recordings from CA1 pyramidal neurons in the hippocampus demonstrated that ethanol (50 mM) increased the frequency but not amplitude of $GABA_A$ sIPSCs (Carta et al., 2003). Additionally, ethanol enhanced the function of presynaptic $GABA_B$ autoreceptors which masked the ethanol-enhancement of $GABA_A$ IPSCs on CA1 pyramidal neurons (Ariwodola and Weiner, 2004). Notably, ethanol (80 mM) enhanced both the frequency and amplitude of $GABA_A$-mediated sIPSCs, however, only the frequency was further potentiated in the presence of a $GABA_B$ receptor antagonist. Acute ethanol (100 mM) application also increased the frequency of $GABA_A$ mIPSCs recorded from pyramidal neurons, thus suggesting that ethanol acts on some presynaptic site to directly facilitate GABA release from these terminals (Sanna et al., 2004). The authors also observed a biphasic effect of ethanol on mIPSC amplitude that showed some developmental interactions. Ethanol (60 mM) increased the frequency of sIPSCs from CA1 neurons more so in adult versus juvenile rats, however there is no age discrepancy regarding ethanol enhancement of mIPSC frequency (Li et al., 2006). In CA1 neurons and dentate gyrus granule cells low concentrations of ethanol (30 mM) had no effect on sIPSC frequency or amplitude (Wei et al., 2004). Overall, at high

concentrations, ethanol application reliably enhances GABA release, however, in only a few studies are there any noticeable effects on postsynaptic $GABA_A$ receptor sensitivity.

1-2-5-1-2-2. Amygdala

The amygdala is also part of the mesolimbic system and serves as a crucial brain region involved in the emotive states associated with drug use and abuse (Koob et al., 1998). In a mechanically isolated neuron/bouton preparation from the BLA, ethanol potentiated action potential-independent GABA release with no effect on postsynaptic $GABA_A$ receptor function (Zhu and Lovinger, 2006). In the rat central nucleus of the amygdala (CeA), ethanol (11-66 mM) potentiated GABAergic transmission at both pre- and postsynaptic sites (Roberto et al., 2003). Unlike in the hippocampus (Ariwodola and Weiner, 2004) and the BLA (Silberman et al., 2009), ethanol enhancement of GABA release in the CeA was not modulated by $GABA_B$ receptor activation. Microdialysis studies have shown an increase in dialysate GABA levels in the CeA after *in vivo* ethanol administration in both naïve and chronic ethanol treated rats (Roberto et al., 2004). The mechanism for ethanol enhancement of GABA release in the mouse CeA is believed to be mediated via presynaptic corticotropin-releasing factor-1 (CRF1) receptors. Perfusion of CRF and ethanol onto CeA neurons produced a decrease in the PPR of evoked IPSCs, thus indicating an increase in GABA release (Nie et al., 2004). These effects were blocked in CRF1 knock-out mice and in wild-type mice in the presence of CRF1 antagonists. Another study

confirmed these findings demonstrating that ethanol and CRF application enhanced the frequency, but not amplitude, of $GABA_A$ mIPSCs and this was blocked with a CRF1 receptor antagonist (Nie et al., 2009). Since CRF and GABA co-localize in these neurons, ethanol may be acting to increase CRF release or CRF1 receptor activation which then enhances GABA release. Additionally, protein kinase C (PKC) ε is an integral factor in the CRF1 receptor-mediated increase in GABA release in the CeA (Bajo et al., 2008). Lastly, ethanol enhancement of GABA release in the CeA is also regulated by delta-opioid receptors (DORs) (Kang-Park et al., 2007). Ethanol enhancement of GABA release in DOR knock-out mice or in the presence of DOR antagonists was greater than in wild-type mice. Thus, endogenous opiate release negatively modulates spontaneous and ethanol-induced GABA release in the CeA.

1-2-5-1-2-3. Cerebellum

The cerebellum plays an important role in coordination and motor control and the ataxic effects of intoxication may be attributable to ethanol actions in this brain region. Ethanol (20-100 mM) increased GABA release, both phasic and tonic, from Golgi cells onto cerebellar granule cells via an enhancement in the firing rate of the Golgi cells (Carta et al., 2004). However, there was no change in sIPSC or evoked IPSC amplitude in the presence of ethanol, suggesting a lack of postsynaptic $GABA_A$ receptor sensitivity to ethanol. Additionally, ethanol (25-100 mM) enhanced mIPSC frequency, but not amplitude, as recorded from cerebellar Purkinje neurons (Ming et al., 2006). When both GABA and glutamate

signaling were blocked however, ethanol enhanced the firing rate of Purkinje neurons. Taken together, these findings suggest that ethanol has dual actions to increase inhibitory drive while also increasing postsynaptic excitability. Ethanol acts directly at the presynaptic terminals synapsing onto Purkinje neurons to enhance GABA release. The ethanol enhancement of GABA release was shown to be dependent on calcium release from ryanodine and inositol-1,4,5-triphosphate- (IP_3) sensitive intracellular calcium stores (Kelm et al., 2007) as well as activation of protein kinase A (PKA) (Kelm et al., 2008). Overall, ethanol enhances GABA release, albeit via different mechanisms, onto cerebellar neurons with no discernible effect on postsynaptic $GABA_A$ receptor sensitivity.

1-2-5-1-2-4. VTA and SN

Given the important role of the midbrain in alcohol addiction, there are surprisingly few studies exploring the role of ethanol modulation of GABA release in the VTA. The first study to measure the effect of ethanol on GABA release in this brain region demonstrated that 24 hours after a single injection of ethanol in mice, there was a long-lasting potentiation of GABA release that is believed to be a manifestation of a long-lasting effect of acute ethanol (Melis et al., 2002; Wanat et al., 2008). Conversely, Ye and colleagues recently reported that ethanol (10-40 mM) may have differential effects on VTA action potential-dependent GABA release. Although under control conditions ethanol decreased GABA release, an increase in sIPSC frequency was observed in the presence of saturating concentrations of the MOR agonist DAMGO (Xiao and Ye, 2008). Thus DAMGO

application may silence VTA interneurons via activation of somatic and dendritic MORs thereby unmasking a separate stimulatory effect of ethanol on other GABAergic inputs. Ye and colleagues also show that ethanol decreased the firing rate of GABA interneurons (Xiao et al., 2007). Steffensen and colleagues demonstrated an ethanol inhibition of interneuron excitability believed to be mediated through inhibition of NMDA receptors (Stobbs et al., 2004). However, they also demonstrated that low dose intravenous ethanol (0.01-0.03 g/kg) enhanced VTA-GABA neuron firing rate in rats (Steffensen et al., 2009). The authors contend that in the latter study, ethanol enhanced dopamine release which then acted to excite local GABA interneurons. In the substantia nigra pars reticulata (SNr), ethanol (100 mM) enhanced $GABA_A$ mIPSC frequency (Criswell et al., 2008). They also observed that a lower dose (50 mM) significantly increased mIPSC frequency in the presence of a $GABA_B$ antagonist, suggesting that, like in the hippocampus (Ariwodola and Weiner, 2004), negative feedback by $GABA_B$ receptor-mediated auto-inhibition can modulate the excitatory effect of ethanol at these terminals. Thus, conflicting results regarding the effects of ethanol on GABA release in the VTA demands the need for further investigation.

1-2-5-1-2-5. Other brain regions

Ethanol has been demonstrated to enhance GABA release onto medium spiny neurons in the NAc (Weiner and Valenzuela, 2006), spinal cord motoneurons (Cheng et al., 1999; Ziskind-Conhaim et al., 2003) and brain stem motoneurons (Sebe et al., 2003). However, ethanol did not enhance GABA

release in the thalamus (Jia et al., 2008), lateral septum (Criswell et al., 2008) or cortex (Moriguchi et al., 2007; Criswell et al., 2008).

1-2-5-2. 5-HT_{2C} receptors

Serotonin (5-Hydroxytryptamine, 5-HT) is a widely distributed neurotransmitter throughout the mammalian CNS. 5-HT_1 through 5-HT_4 are the four major receptor families with defined functions, with each family expressing multiple subtypes. 5-HT_1 and 5-HT_4 receptors inhibit and activate adenylyl cyclase, respectively. 5-HT_2 receptors are coupled to PLC activation and generation of IP_3 and diacylglycerol. The 5-HT_3 receptor is unique in being the only monoamine neurotransmitter receptor that functions as a ligand-gated ion channel permeable to both Na^+ and K^+ ions. The 5-HT_{2C} receptor (5-HT_{2C}R) subtype is a G_q protein-coupled receptor that facilitates PLC-mediated IP_3 accumulation and release of calcium from intracellular stores (Conn et al., 1986). This receptor is predominantly expressed in the choroid plexus but is also found in the midbrain with evidence showing 5-HT_{2C}R mRNA localized to GABAergic neurons of the SN (Eberle-Wang et al., 1997). Additionally, this receptor is expressed on both synaptic terminals and cell soma of VTA-GABA neurons of the VTA though it has also been reported to occur on DA neurons as well (Bubar and Cunningham, 2007).

Serotoninergic afferents from the midbrain raphe nuclei innervate both DA and GABAergic neurons of the VTA (Herve et al., 1987) and thus may influence DA output from the VTA. Indeed, electrophysiological evidence indicates that 5-

HT reuptake blockade inhibits VTA-DA firing suggesting that a predominant action of 5-HT in the VTA is inhibitory and may involve activation of GABAergic neurons (Di Mascio et al., 1998). This contention is supported by the observations that 5-HT_{2C}R activation inhibits VTA-DA neuron firing (Di Matteo et al., 2000) and decreases DA output to the frontal cortex, striatum and NAc (Gobert et al., 2000). Activation of the IP_3-calcium pathway on GABAergic soma or terminals may result in enhancement of GABA release. Methylenedioxymethamphetamine ('ecstasy') increases GABA release in the VTA through a 5-HT_{2C}R-dependent mechanism (Bankson and Yamamoto, 2004). Additionally, local and systemic administration of the 5-HT_{2C}R agonist Ro-60-0175 was observed to stimulate GABA release in the SN (Invernizzi et al., 2007). Furthermore, activation of these receptors in the dorsal raphe nucleus inhibits 5-HT containing neurons via activation of local GABAergic neurons (Boothman et al., 2006).

1-2-5-2-1. Ethanol and 5-HT

No *in vitro* electrophysiological studies have previously explored the role of 5-HT_{2C}Rs specifically in the actions of ethanol. However, 5-HT_{2C}Rs may exert tonic control over the motivational aspects of ethanol drinking behavior. One study found that activation of 5-HT_{2C}Rs decreased ethanol self-administration whereas inhibition of these receptors had the opposite effect (Tomkins et al., 2002). Furthermore, ethanol increased 5-HT release in the NAc (Yoshimoto et al., 1992) and prevented 5-HT reuptake in the hippocampus (Daws et al., 2006).

Thus, it is apparent that there is a 5-HT/ethanol interaction in the CNS and given the modulatory role of 5-HT_{2C}Rs in the midbrain, further investigation is necessary to uncover a potential ethanol interaction in the VTA.

1-2-5-3. Opioid receptors

Opioid receptors are GPCRs with multiple transduction mechanisms. Three known classes of opioid receptors include mu, delta and kappa which show high affinity for the endogenous opioids beta-endorphin, enkephalin and dynorphin, respectively (Zollner and Stein, 2007). All three receptors exhibit generally similar mechanisms in that they are coupled to $G_{i/o}$ proteins. These receptors exhibit multiple functions and have been demonstrated to inhibit neurotransmitter release (Schoffelmeer et al., 1988), inhibit adenylyl cyclase (Sharma et al., 1977), activate K^+-dependent calcium channels (North et al., 1987), stimulate PLC (Murthy and Makhlouf, 1996) and inhibit VGCCs (Morikawa et al., 1995). MORs are localized presynaptically to GABAergic terminals in the VTA (Svingos et al., 2001) and activation of these receptors disinhibits DA neurons (Johnson and North, 1992a; Margolis et al., 2003). This is likely due to a reduction in GABA release via MOR-mediated inhibition of the secretory process at the nerve terminal of GABAergic cells (Bergevin et al., 2002). KORs are also located in the VTA and activation of these receptors directly inhibits DA neuron activity (Margolis et al., 2003). Additionally, activation of MORs and KORs in the VTA inhibits glutamatergic input onto both DA and GABAergic neurons (Margolis et al., 2005). Activation of DORs in the VTA decreases

frequency, but not amplitude of m/sIPSCs recorded from VTA-DA neurons thus suggesting that DORs are also located presynaptically on GABAergic terminals (Margolis et al., 2008). It should be noted however, this effect is only seen in drinking animals and not in ethanol-naïve animals.

1-2-5-3-1. Ethanol and opioids

The rewarding and reinforcing properties of ethanol have been linked to various opiate receptor subtypes in the mesolimbic system. Acute ethanol exposure has been demonstrated to increase endogenous beta-endorphin levels in a variety of brain structures (Gianoulakis and Barcomb, 1987; de Waele and Gianoulakis, 1993; Olive et al., 2001) including the VTA (Rasmussen et al., 1998; Jarjour et al., 2009). Ethanol also increases levels of enkephalins and dynorphins but results between studies are inconsistent (Jarjour et al., 2009).

Numerous behavioral studies suggest that the opioidergic system modulates ethanol drinking behavior. Naltrexone and naloxone, two antagonists with affinity to all three opioid receptor subtypes, reduce ethanol consummatory behaviors (Gonzales and Weiss, 1998; Shoemaker et al., 2002; Stromberg et al., 2002). However, there is more consistent evidence demonstrating MOR modulation of ethanol intake behaviors. The use of selective MOR antagonists or genetic deletion of MORs reduces ethanol drinking (Honkanen et al., 1996; Krishnan-Sarin et al., 1998; Stromberg et al., 1998; Kim et al., 2000; Hall et al., 2001; Hyytia and Kiianmaa, 2001; Mhatre and Holloway, 2003; Lasek et al.,

2007; Margolis et al., 2008); however, these results are not universal (Le et al., 1993).

The precise role of DORs and KORs in ethanol consummatory behaviors is less clear with variable results across studies and animal models used. For example, some studies have demonstrated a lack of an effect of DOR antagonism on ethanol intake (Honkanen et al., 1996; Stromberg et al., 1998; Ingman et al., 2003). Alternatively, other studies have shown that antagonism of DORs reduced ethanol intake (Le et al., 1993; Krishnan-Sarin et al., 1995; June et al., 1999; Kim et al., 2000; Hyytia and Kiianmaa, 2001). DOR antagonism was recently reported to produce a long-lasting decrease in ethanol consumption in high- but not low-ethanol drinking rats (Nielsen et al., 2008). Conversely, one study demonstrated that DOR antagonism in the VTA increased ethanol consumption in low-drinking rats whereas activation reduced consumption (Margolis et al., 2008). The authors contend that chronic ethanol consumption upregulates DOR function which acts to decrease GABA release onto DA neurons. There is general agreement that KOR activation decreases ethanol drinking (Sandi et al., 1988; Nestby et al., 1999; Lindholm et al., 2001); however, some studies have shown KOR knock-out mice display decreased drinking (Kovacs et al., 2005) and KOR antagonism reduced ethanol drinking in ethanol-dependent rats only (Walker and Koob, 2008). Additionally, mice lacking preprodynorphin, the precursor to dynorphins which selectively bind KORs, show reduced ethanol consumption (Blednov et al., 2006).

The reason for the variability between studies is unclear; however, further work with more selective opioid receptor antagonists may help to delineate the precise roles of the different receptors in the motivational responding and reinforcing properties of ethanol drinking. Additionally, variability with species and strain of the animals used in addition to the method and location of drug delivery could explain some of the differences between studies. In spite of this, there does appear to be strong consensus in the role of MORs in mediating some of the reinforcing properties of ethanol.

Modulation of DA release to the NAc may explain the effect of opioids to regulate ethanol consummatory behaviors. Ethanol intake increases the output of VTA-DA neurons, and this output is concurrently modulated by opioidergic systems. Recent evidence suggests that the ethanol-induced increase in dopamine release to the NAc is modulated by MORs (Job et al., 2007). The authors demonstrated that pharmacological blockade with the MOR-selective antagonist, naloxonazine, or genetic deletion of MORs reduced the ethanol-enhancement in DA release, suggesting that MORs positively regulate DA activity in the VTA. Indeed, MORs are localized presynaptically to GABAergic terminals in the VTA (Svingos et al., 2001) and activation of these receptors disinhibits DA neurons (Johnson and North, 1992a; Margolis et al., 2003). In light of this evidence, ethanol may act to increase endorphin release into the VTA and thus disinhibit DA neurons thereby constituting a potential mechanism in MOR regulation of ethanol drinking. Interestingly, in the CeA, ethanol increased

GABA release and this effect was enhanced in DOR knock-out mice and in the presence of DOR antagonists (Kang-Park et al., 2007). That study suggests that endogenous opiate release negatively modulates spontaneous and ethanol-induced GABA release in the CeA; thus, a similar scenario may exist in the VTA and may thus have implications for the therapeutic utility of naltrexone.

1-3. Calcium and neurotransmitter release

1-3-1. Role of extracellular calcium and VGCCs

Action potential propagation down to the presynaptic terminal activates VGCCs near neurotransmitter release machinery and the subsequent rise in local calcium concentration in this region activates a cascade of events resulting in the release of neurotransmitter quanta. N- and P/Q-type channels are those predominantly involved in transmitter release (Wheeler et al., 1994), although L-type channels have been reported to regulate spontaneous GABA release as well (Rhee et al., 1999). Although quantal neurotransmitter release is typically measured electrophysiologically in the presence of tetrodotoxin (TTX), some neurons express TTX-insensitive Na^+ channels that mediate a subthreshold depolarizing current that may be capable of activating VGCCs allowing for calcium influx. A TTX-insensitive current is found in GABAergic neurons of the SNr (Atherton and Bevan, 2005) and could exist in VTA, thus the resultant depolarization may lead to activation of VGCCs.

1-3-1-1. Ethanol and VGCCs

It is widely accepted that ethanol inhibits some VGCCs and this may impart some of the physiological effects of ethanol intoxication (Walter and Messing, 1999). Ethanol decreased calcium influx through VGCCs in cultured superior cervical ganglion (SCG) neurons (Xiao et al., 2005). In pheochromocytoma (PC12) cells, ethanol inhibited dihdyropyridine-sensitive L-type VGCCs but had little to no effect on ω-conotoxin-sensitive N-type VGCCs (Mullikin-Kilpatrick and Treistman, 1995). In the hippocampus, ethanol was shown to inhibit glutamate release via inhibition of N/P/Q-type VGCCs (Maldve et al., 2004) as well as L-type VGCC mediated synaptic plasticity of excitatory transmission (Hendricson et al., 2003). Ethanol has also been demonstrated to inhibit voltage-gated Na^+ channels (VGSCs) in cultured SCG neurons (Xiao et al., 2008) and recombinant VGSCs expressed in *xenopus* oocytes, specifically those channels containing the $Na_v1.2$ α-subunit (Shiraishi and Harris, 2004).

1-3-2. Role of IP_3- and ryanodine-sensitive intracellular calcium stores

Ryanodine- and IP_3-sensitive intracellular calcium stores play a role in regulating transmitter release in several mammalian neurons. Ryanodine-sensitive calcium stores modulate both action potential-dependent and independent GABA release from presynaptic nerve terminals in the cerebellum (Llano et al., 2000; Galante and Marty, 2003) and spontaneous GABA release in the hippocampus of neonates (Savic and Sciancalepore, 1998). In hippocampal presynaptic boutons, ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release (CICR)

controls approximately half of all transmitter release (Emptage et al., 2001). Presynaptic calcium stores also contribute to GABA release onto cerebellar Purkinje neurons in the mouse (Bardo et al., 2002). In retinal amacrine cells, calcium released from IP_3-sensitive stores, but not ryanodine, controls spontaneous transmitter release (Warrier et al., 2005). Likewise, spontaneous GABA release onto Meynert neurons in the rat are partially influenced by IP_3-senstive calcium stores (Rhee et al., 1999). In the striatum, dopamine transmission is modulated by CICR through IP_3-sensitive calcium stores (Zhu et al., 2004). Ryanodine and IP_3 receptor-dependent calcium mobilization has been demonstrated in DA neurons of the VTA in rats (Morikawa et al., 2003). Furthermore, ryanodine receptors are present in GABAergic nerve terminals of the SNr, a neighboring region of the VTA (Yanovsky et al., 2005).

1-3-2-1. Ethanol and intracellular calcium stores

Although typically reducing the influx of calcium through VGCCs, ethanol stimulates calcium release from intracellular stores. Ethanol increased calcium release from IP_3-senstive stores in brain microsomes (Daniell and Harris, 1989). In cultured hippocampal neurons ethanol increased calcium release from a single pool containing both ryanodine and IP_3 receptors (Mironov and Hermann, 1996). Ethanol dose-dependently increased basal intracellular calcium levels in primary cultured SCG neurons as well (Xiao et al., 2005). Most importantly, calcium release from presynaptic IP_3- and ryanodine-sensitive internal stores is

necessary for ethanol potentiation of GABA release onto Purkinje neurons in the cerebellum (Kelm et al., 2007; Kelm et al., 2008).

1-4. Hypothesis and Aims

Ethanol enhances opioid release in the VTA and resultant activation of MORs present on GABAergic interneurons should theoretically disinhibit VTA-DA neurons. Disinhibition via a decrease in GABA release could be one mechanism by which ethanol enhances VTA-DA neuron activity. However, preliminary data demonstrated an ethanol-enhancement in GABA release onto VTA-DA neurons. Therefore, the overarching hypothesis of the current proposal states that ethanol enhancement of GABA transmission in the VTA may be important for modulating alcohol drinking behavior and alcohol dependence. Two working hypotheses arise from this overarching hypothesis and preliminary work and will be investigated here: (1) ethanol-enhanced GABA release is mediated via release of calcium from internal stores through a 5-HT_{2C} receptor-dependent mechanism and (2) the ethanol-induced increase in GABA release limits the stimulatory effect of ethanol on VTA-DA firing rate and is masked by endogenous opioid tone acting on presynaptic MORs. The specific aims are:

Aim 1.1: Investigate the relative role of extracellular calcium on the ethanol-induced enhancement in GABA release. Utilizing whole-cell voltage-clamp recordings of mIPSCs from VTA-DA neurons, we will investigate the role of extracellular calcium on ethanol-elicited GABA release in the VTA.

Aim 1.2: To test the hypothesis that ethanol-enhancement in presynaptic GABA release is mediated via calcium release from intracellular stores. Utilizing whole-cell voltage clamp recordings of mIPSCs from VTA-DA neurons, we will investigate the role of intracellular calcium stores on ethanol-elicited GABA release.

Aim 1.3: To test the hypothesis that ethanol increases synaptic GABA release through a 5-HT_{2C} receptor-dependent mechanism. Utilizing whole-cell voltage-clamp recordings of mIPSCs from VTA-DA neurons, we will investigate the role of the 5-HT_{2C} receptor in ethanol-elicited GABA release in the VTA.

Aim 2.1: To test the hypothesis that the ethanol-induced increase in synaptic GABA release limits the ethanol-enhancement in VTA-DA neuron activity. Utilizing cell attached current-clamp recordings we will investigate $GABA_A$ receptor-dependent regulation of VTA-DA neuron firing rate and interactions with ethanol.

Aim 2.2: To test the hypothesis that the ethanol-induced increase in synaptic GABA release is modulated by presynaptic μ-opiate receptors. Utilizing a combination of whole-cell voltage-clamp and cell attached current-clamp recordings we will investigate MOR-dependent regulation of GABA release, VTA-DA neuron activity and interactions with ethanol.

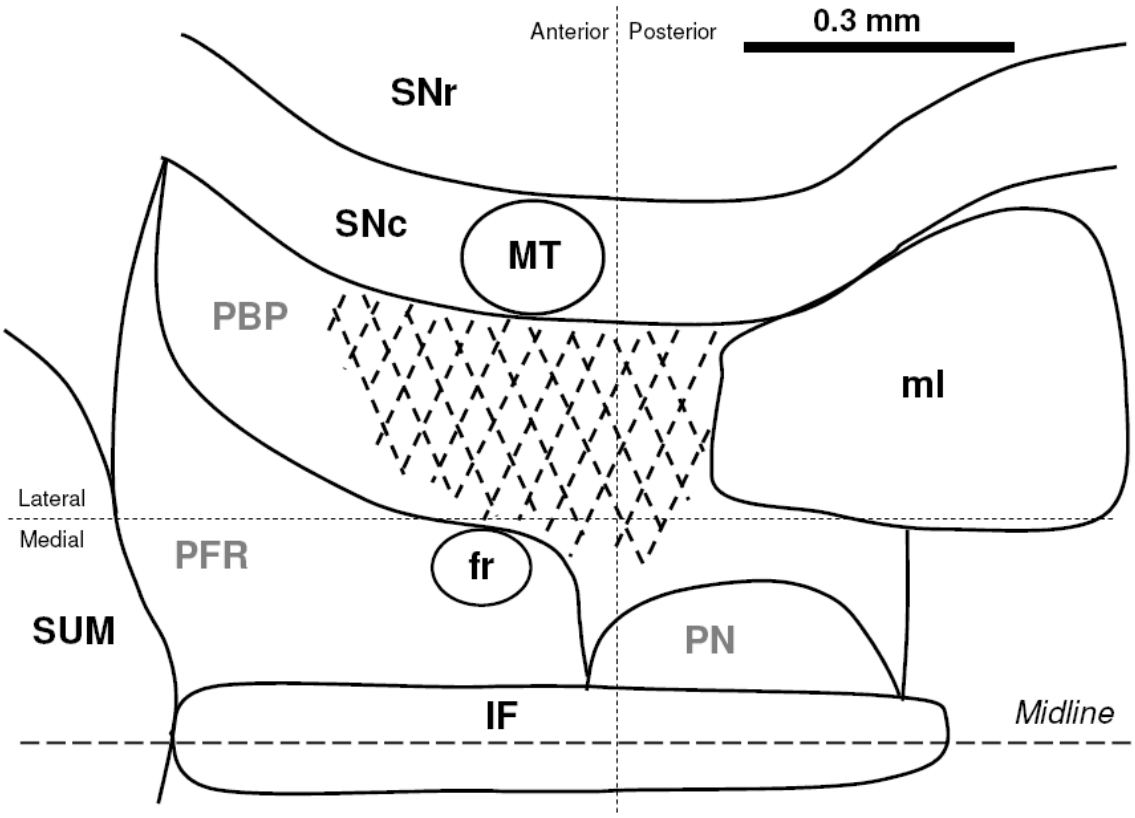


Figure 1-1. Areas of VTA in which recordings were conducted. A diagram representing the approximate location (represented by hatched area) of VTA-DA recordings for the experiments of this dissertation. Abbreviations: IF, interfascicular nucleus; ml, medial lemniscus; fr, fasciculus retroflexus; MT, medial terminal nucleus of the accessory optic tract; PBP, parabrachial pigmented area; PFR, parafasciculus retroflexus area; PN, paranigral nucleus; SNc, substantia nigra compacta; SNr, substantia nigra reticulata; SUM, supramammillary nucleus. The PBP, PN and PFR are subregions of the VTA. In an adolescent rat (21-28 days postnatal), the dorsal-ventral plane is approximately -4.8 mm from bregma; the anterior-posterior plane is approximately -3.3 mm from bregma.

Chapter 2: Ethanol Enhances GABAergic Transmission onto Dopamine Neurons in the Ventral Tegmental Area of the Rat

Background: Activation of the dopaminergic (DA) neurons of the ventral tegmental area (VTA) by ethanol has been implicated in its rewarding and reinforcing effects. At most central synapses ethanol generally increases inhibitory synaptic transmission, however no studies have explored the effect of acute ethanol on GABAergic transmission in the VTA.

Methods: Whole-cell patch clamp recordings of inhibitory postsynaptic currents (IPSCs) from VTA-DA neurons in midbrain slices from young rats.

Results: Acute exposure of VTA-DA neurons to ethanol (25-50 mM) robustly enhanced GABAergic spontaneous and miniature IPSC frequency while inducing a slight enhancement of sIPSC amplitude. Ethanol (50 mM) enhanced paired-pulse depression of evoked IPSCs further suggesting enhanced GABA release onto VTA-DA neurons. The frequency of sIPSCs was suppressed by the $GABA_B$ agonist, baclofen (1.25 μM) and enhanced by the antagonist, SCH50911 (20

μM); however, neither appeared to modulate or occlude the effects of ethanol on sIPSC frequency.

Conclusions: The present results indicate that ethanol increases postsynaptic $GABA_A$ receptor sensitivity, enhances action potential-independent GABA release onto VTA-DA neurons, and that this effect is independent of $GABA_B$ auto-receptor inhibition of GABA release.

2-1. Introduction

Dopaminergic (DA) neurons of the ventral tegmental area (VTA) constitute the central locus of the mesocorticolimbic dopamine system and send projections to the nucleus accumbens (NAc), prefrontal cortex, basolateral amygdala and a variety of other corticolimbic structures (Albanese and Minciacchi, 1983; Oades and Halliday, 1987). Substantial evidence suggests these VTA-DA projections contribute to certain aspects of drug reinforcement and/or reward (Gatto et al., 1994; Robbins and Everitt, 1996; Wise, 1996; Koob et al., 1998; Schultz, 2002; Appel, 2004). Indeed, ethanol has been shown to increase the firing rate of DA neurons in the VTA both *in vitro* (Brodie et al., 1990) and *in vivo* (Gessa et al., 1985). Ethanol may be acting through direct effects on DA neuron excitability (Brodie and Appel, 1998; Brodie et al., 1999b) that are at least partly attributable to an increase in the hyperpolarization-activated cation current (I_h) (Okamoto et al., 2006).

In addition to the principal DA neurons, the VTA also contains a major population of GABAergic neurons (Grace and Onn, 1989; Johnson and North,

1992b; Korotkova et al., 2003). The GABAergic neurons function importantly as local interneurons that regulate the firing of the DA neurons via tonic inhibition (Johnson and North, 1992a; Bonci and Malenka, 1999). The effect of acute ethanol on GABAergic transmission has been extensively explored in a variety of brain regions (Wan et al., 1996; Roberto et al., 2003; Carta et al., 2004; Nie et al., 2004; Weiner and Valenzuela, 2006); however, remarkably, virtually nothing is known about the direct effects of acute ethanol on GABAergic transmission in the VTA. One electrophysiological study measured prolonged enhancement of GABAergic transmission in mice 24 hours after a single *in vivo* exposure to ethanol (Melis et al., 2002).

Thus while excitatory actions of ethanol in the VTA have been well-documented, the possibility that conventional stimulatory effects of ethanol on GABAergic transmission that also may modulate VTA-DA neuronal excitability has not been investigated. To determine whether ethanol modulates GABAergic transmission in this brain region, we studied $GABA_A$-mediated IPSCs recorded from VTA-DA neurons using conventional whole-cell patch clamp techniques in the presence of varying concentrations of ethanol.

2-2. Materials and Methods

2-2-1. Slice preparation

All experiments were carried out in accordance with NIH guidelines. Slices used in this study were prepared from Sprague-Dawley rats of both sexes (postnatal day 21 to 30). Rats were anesthetized with halothane, decapitated,

and the brain was rapidly removed and placed in ice-cold, oxygenated artificial cerebrospinal buffer (aCSF) containing (in mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KCl, 1.23 NaH_2PO_4 and 10 dextrose, 95%O_2/5%CO_2 (all chemicals obtained from Sigma, St Louis, MO). Cutting aCSF also contained 2.4 mM $MgSO_4$ and 1.8 mM $CaCl_2$. Horizontal midbrain slices (200-220 μm) were prepared using a vibratome (VT1000S; Leica, Nussloch, Germany). The slices were then maintained at 32°C in cutting aCSF buffer for a minimum of 60 minutes before electrophysiological recordings were performed.

2-2-2. Electrophysiological recordings

Slices were transferred to a recording chamber and perfused with oxygenated aCSF (30-32°C) at a flow rate of ~2 ml/min. Recording aCSF also contained 0.9 mM $MgSO_4$ and 2 mM $CaCl_2$. DA neurons were identified by the presence of a large hyperpolarization-induced I_h current (>200 pA) that was assayed immediately following break-in by application of a 1.5-s hyperpolarizing step from -60 to -110 mV (Johnson and North, 1992b). Only DA neurons identified in this manner from the VTA were used for this study. Recording electrodes were made from thin-walled borosilicate glass (TW 150F-4, WPI, Sarasota, Florida, 1.5-2.5 MΩ). Recording electrodes for whole-cell recordings contained (in mM): 135 KCl, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Tris-GTP, pH 7.3 with KOH (all chemicals obtained from Sigma, St Louis, MO). Access resistance was partially compensated and monitored throughout all experiments. Data were collected by an Axon Instruments Model 200B amplifier

filtered at 1 kHz and digitized at 10-20 kHz with a Digidata interface using pClamp v9.2 (Axon Instruments, Foster City, California).

Kynurenic acid (1 mM) was used in all whole-cell voltage clamp recordings to inhibit AMPA- and NMDA receptor-mediated currents. All GABA IPSCs were inward at a holding potential of -60 mV and completely blocked by application of picrotoxin (50 µM). Miniature IPCSs (mIPSCs) were recorded in the presence of 0.5 µM tetrodotoxin (TTX) to block action potentials. Spontaneous IPSCs (sIPSCs) were recorded in the absence of TTX. Following break-in and after a stable 5-10 minute baseline (control) recording, drugs were bath-applied through the aCSF and a continuous 5-15 minute recording was made to detect any changes in m/sIPSC frequency and amplitude. The number of neurons used per each treatment condition is represented as *n* with only one neuron used per slice. A five minute drug wash-on preceded the start of data collection in each treatment condition. A 10-15 minute washout period followed drug application. SCH50911 was obtained from Tocris Bioscience, Ellisville, MO. Baclofen, kynurenic acid, and picrotoxin were obtained from Sigma, St. Louis, MO. TTX was obtained from Alomone Labs, Jerusalem, Israel.

A monopolar tungsten stimulating electrode was placed 50-100 µm rostral of the recording electrode for evoked IPSC recordings. Constant-current pulses (100-µs duration, 15–40 pA amplitude) were applied through a stimulus isolation unit driven by an analog stimulator (WPI). Paired-stimuli were delivered every 20 or 30 sec with an interstimulus interval of 70 msec.

2-2-3. Data analysis

For m/sIPSC recordings, quantal events were detected (60-90 sweeps each condition, 5 sec/sweep) and analyzed using Clampfit (pClamp v9.2 software; Axon Instruments). Access resistance for most experiments ranged from 6 to 20 MΩ and never exceeded 25 MΩ. To maintain the fidelity of the recordings, experiments where access resistance changed (>20% at anytime during the experiment) were not included in data analyses. To accurately determine the m/sIPSC amplitude, only those events that were >10 pA were accepted for analysis in order to maintain a high signal-to-noise ratio. Treatment and washout groups were normalized to the baseline (control) frequency or amplitude and represented as a percentage of the control. For evoked paired stimuli, the ratio between the second and the first IPSCs was calculated and averaged for a 10 minute baseline and a minimum 10 minute treatment condition. Averaged values for all data sets are expressed as mean ± SEM and were compared statistically using two-tailed paired or unpaired student's *t* test or ANOVA/Dunnett C or Tukey HSD post-hoc where mentioned. Significant differences were considered as $*p < 0.05$ and $**p < 0.01$.

2-3. Results

2-3-1. Baseline parameters of sIPSCs on VTA neurons

Data in this study were gathered using whole-cell voltage-clamp from VTA neurons that were identified as dopaminergic by the presence of a large I_h current (see *Materials and Methods*). The $GABA_A$ receptor blocker picrotoxin (50

μM) completely blocked the sIPSCs, indicating that they were GABA-mediated (data not shown, *n* = 3). The baseline frequency and amplitude of sIPSCs recorded in control conditions from all cells tested across every treatment group were 3.89 ± 0.30 Hz and 52.01 ± 1.31 pA, respectively (*n* = 52).

2-3-2. Ethanol enhances GABAergic transmission in the VTA

To measure the effect of varying concentrations of ethanol (15- 50 mM; legal intoxication is ~17 mM), $GABA_A$-mediated sIPSCs were recorded across this range of ethanol concentrations in two separate experiments in separate groups of slices: 15 and then 25 mM (Fig. 1), and 25 and then 50 mM (Fig. 2) ethanol. Thus the inherent difficulties of maintaining very long-lasting whole-cell recordings for these concentration-response experiments were minimized. After application of ethanol (15 mM), no significant increases in sIPSC frequency (Fig. 1A, B) and amplitude (Fig. 1A, C) were observed. However, subsequent application of ethanol (25 mM) produced a significant increase in sIPSC frequency (Fig. 1A, B) but not amplitude (Fig. 1A, C).

In the separate cohort of recordings where 25 mM ethanol was initially applied, interesting differences in sIPSC event frequency and amplitude were observed (Fig. 2) compared to the effect presented in the previous 15-25 mM experiment. In this instance, application of ethanol (25 mM) did not result in a significant increase in either sIPSC frequency or amplitude (Figs. 2C, D). However, subsequent application of ethanol (50 mM) to the same neuron produced a significant increase in sIPSC frequency (Fig. 2C) and amplitude (Fig.

2D). The distributions of the sIPSC inter-event intervals and amplitude for control, ethanol (25, 50 mM) and wash conditions are shown in a sample representative neuron (Figure 2E, F). There was a progressive leftward shift in the inter-event interval distribution, indicative of increased GABA release that at least partially reversed upon wash (Fig. 2E). Additionally, there was a progressive rightward shift in peak amplitude distribution, indicative of increased postsynaptic GABA sensitivity, which partially reversed upon wash (Fig. 2F). The cumulative changes in sIPSC frequency and amplitude recorded at each ethanol concentration were pooled from all experiments [15 mM (n = 7), 25 mM (n = 22), and 50 mM ethanol (n = 11)] (Fig. 3). Application of ethanol (25 and 50 mM) produced a significant increase above control in frequency (Fig. 3A) and amplitude (Fig. 3B). Ethanol at 15 mM did not produce a significant increase in either frequency or amplitude. No concentration above 50 mM was tested. It is conceivable that the observed ethanol-induced increase in sIPSC amplitude, although slight, may result in an apparent increase in sIPSC frequency due to the detections of small events under control conditions which were below detection threshold (10 pA) and thus not previously detected. Therefore, we calculated the % enhancement induced by ethanol of the number of events for a defined recording duration binned across all amplitude ranges. As shown in Fig. 4C, from a neuron which showed a maximal increase in sIPSC frequency (~86% above control) ethanol increases the number of events relatively equivalently across all amplitude bins.

To investigate further the ethanol-mediated enhancement in presynaptic release as demonstrated in the increase in sIPSC frequency, we measured the paired-pulse ratio of evoked IPSCs (as described in *Materials and Methods*) before and after application of ethanol (50 mM). In 5 of 6 VTA-DA neurons, application of 50 mM ethanol resulted in a significant enhancement of paired-pulse depression (PPD), indicative of enhanced GABA release (Fig. 4C). Additionally, we measured the decay time of the evoked IPSCs and found that ethanol prolonged the decay time constant, τ, indicative of an enhancement, although slight, in postsynaptic $GABA_A$ receptor sensitivity (Fig. 4D).

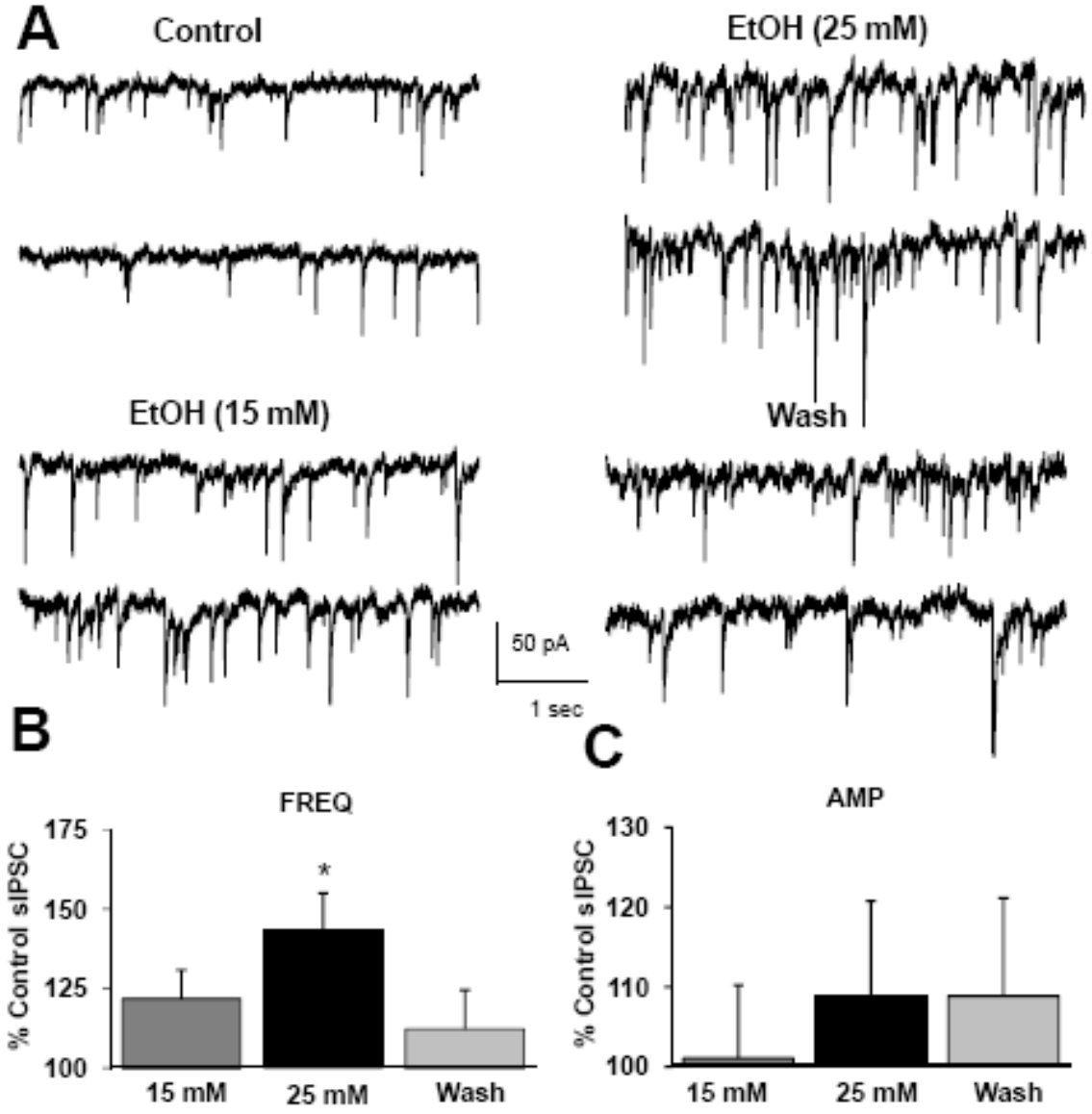


Figure 2-1. Ethanol (25 mM) potentiates sIPSC frequency. A. sIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 15 mM and 25 mM ethanol, and after a washout. B. A bar graph representing the % change ± SEM above control sIPSC frequency for the conditions shown in A. Event frequency under control conditions was 4.62 ± 0.75 Hz. C. A bar graph representing the % change ± SEM above control sIPSC amplitude for the conditions shown in A. Event amplitude under control conditions was 52.82 ± 2.28 pA. (n = 7, * indicates $p < 0.05$ by ANOVA/Dunnett C post-hoc different from control).

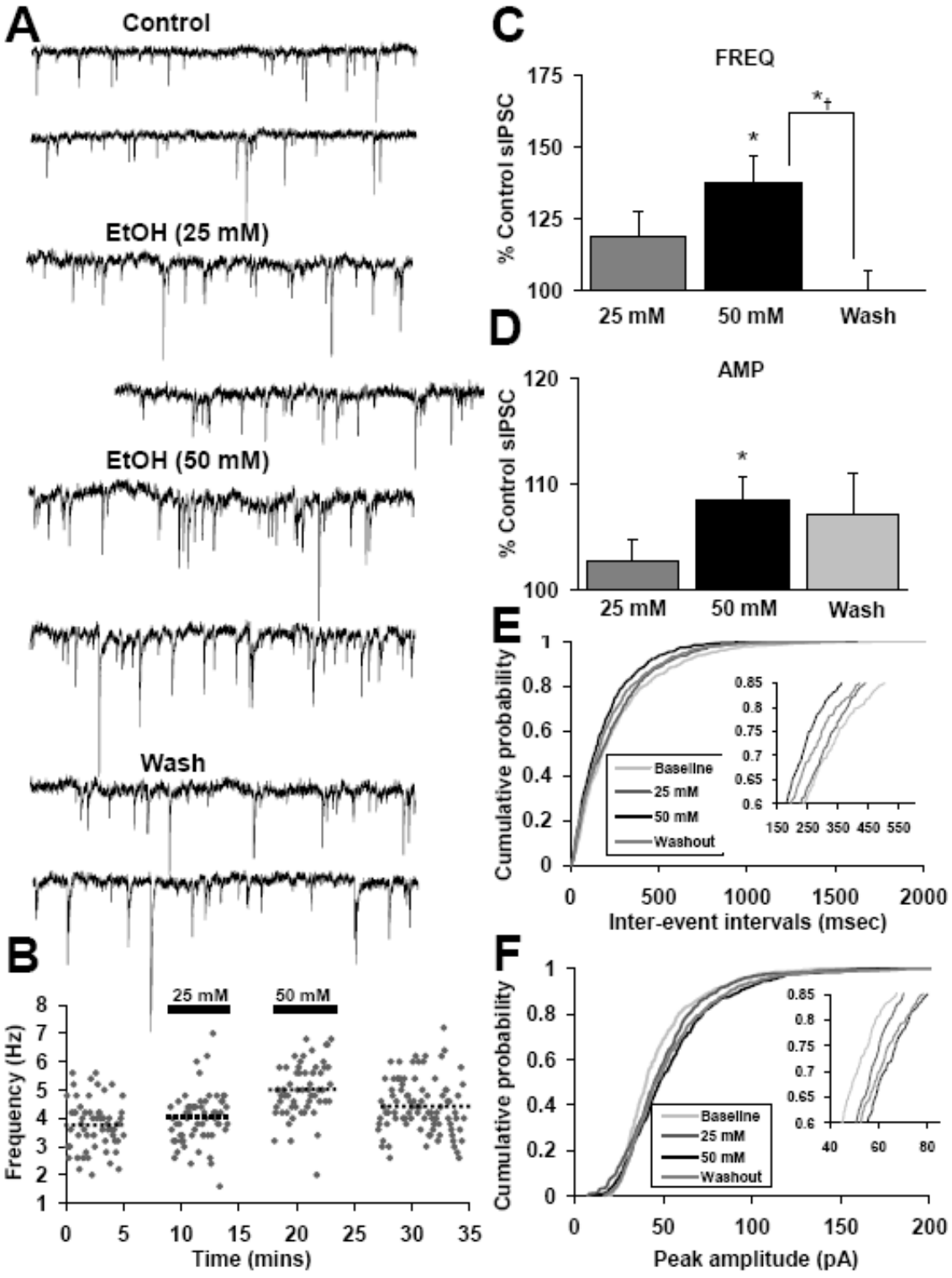

A
Control
EtOH (25 mM)
EtOH (50 mM)
Wash
B
25 mM
50 mM
Frequency (Hz)
Time (mins)
C
FREQ
% Control sIPSC
25 mM
50 mM
Wash
D
AMP
% Control sIPSC
25 mM
50 mM
Wash
E
Cumulative probability
Baseline
25 mM
50 mM
Washout
Inter-event intervals (msec)
F
Cumulative probability
Baseline
25 mM
50 mM
Washout
Peak amplitude (pA)

Figure 2-2. Ethanol (50 mM) potentiates sIPSC frequency and amplitude. A. sIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 25 mM and 50 mM ethanol, and after a washout. B. Time course for a sample representative neuron under conditions shown in A. The dotted lines represent the average frequency under each condition for that neuron. C. A bar graph representing the % change $\pm$ SEM above control sIPSC frequency for the conditions shown in A. Event frequency under control conditions was 5.48 $\pm$ 0.93 Hz. D. A bar graph representing the % change $\pm$ SEM above control sIPSC amplitude for the conditions shown in A. Event amplitude under control conditions was 54.49 $\pm$ 5.56 pA. E. Cumulative probability histogram of sIPSC inter-event intervals for epochs from same neuron shown in B. F. Cumulative probability histogram of sIPSC event amplitudes from the same neuron shown in B, E. (n = 8, * indicates $p < 0.05$ by ANOVA/Dunnett C post-hoc different from control, *† indicates $p < 0.05$ by ANOVA/Dunnett C post-hoc different from wash).

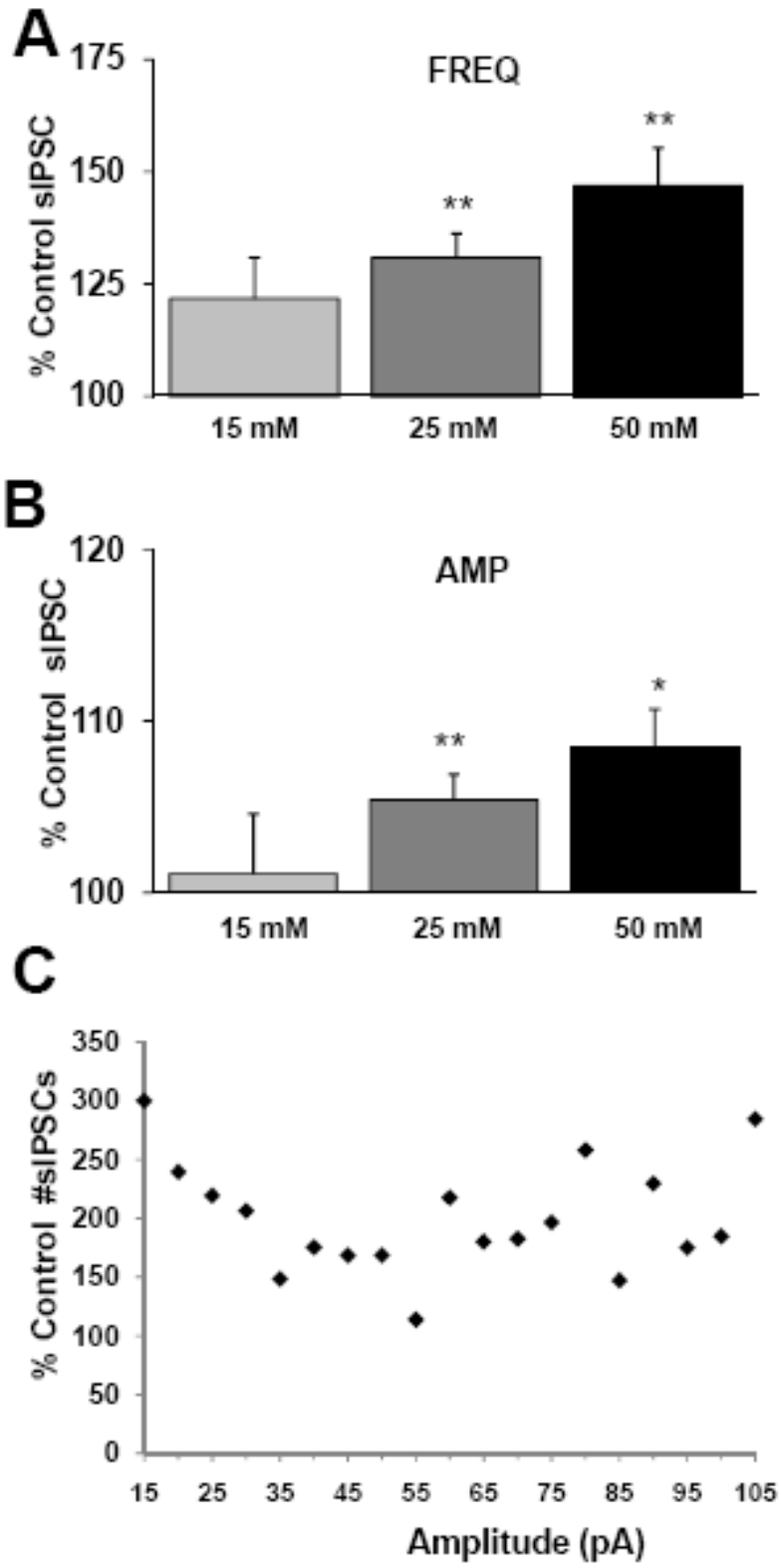

A
FREQ
% Control sIPSC
175
150
125
100
**
**
15 mM
25 mM
50 mM
B
AMP
% Control sIPSC
120
110
100
**
*
15 mM
25 mM
50 mM
C
% Control #sIPSCs
350
300
250
200
150
100
50
0
15
25
35
45
55
65
75
85
95
105
Amplitude (pA)

Figure 2-3. Cumulative results show ethanol-induced potentiation of sIPSC frequency and amplitude at varying concentrations. A. A bar graph representing the % change ± SEM above control sIPSC frequency in the presence of 15 mM, 25 mM, and 50 mM ethanol. Event frequency under control conditions was 4.92 ± 0.44 Hz. B. A bar graph representing the % change ± SEM above control sIPSC amplitude for the conditions shown in A. Event amplitude under control conditions was 52.92 ± 2.06 pA. (n = 7 for 15 mM, n = 22 for 25 mM, n = 11 for 50 mM, * indicates $p < 0.05$, ** indicates $p < 0.01$ by ANOVA/Dunnett C post-hoc different from control) C. A sample cell was analyzed that showed a maximal ethanol-induced (50 mM) enhancement in sIPSC frequency (~86% above control) to determine the percent change in the number of events at their respective amplitude distribution. The events were binned into 5 pA groups, for example, the point at 30 pA represents all events >25 and <30 pA. All events at 105 pA represent those events greater than 100 pA in size. The total number of events was 1,148 and 2,135 in control and ethanol conditions, respectively, taken from a time course of 5 minutes for each condition. (Note: the 300% change in events <15 pA represents an increase from only 4 to 12 events).

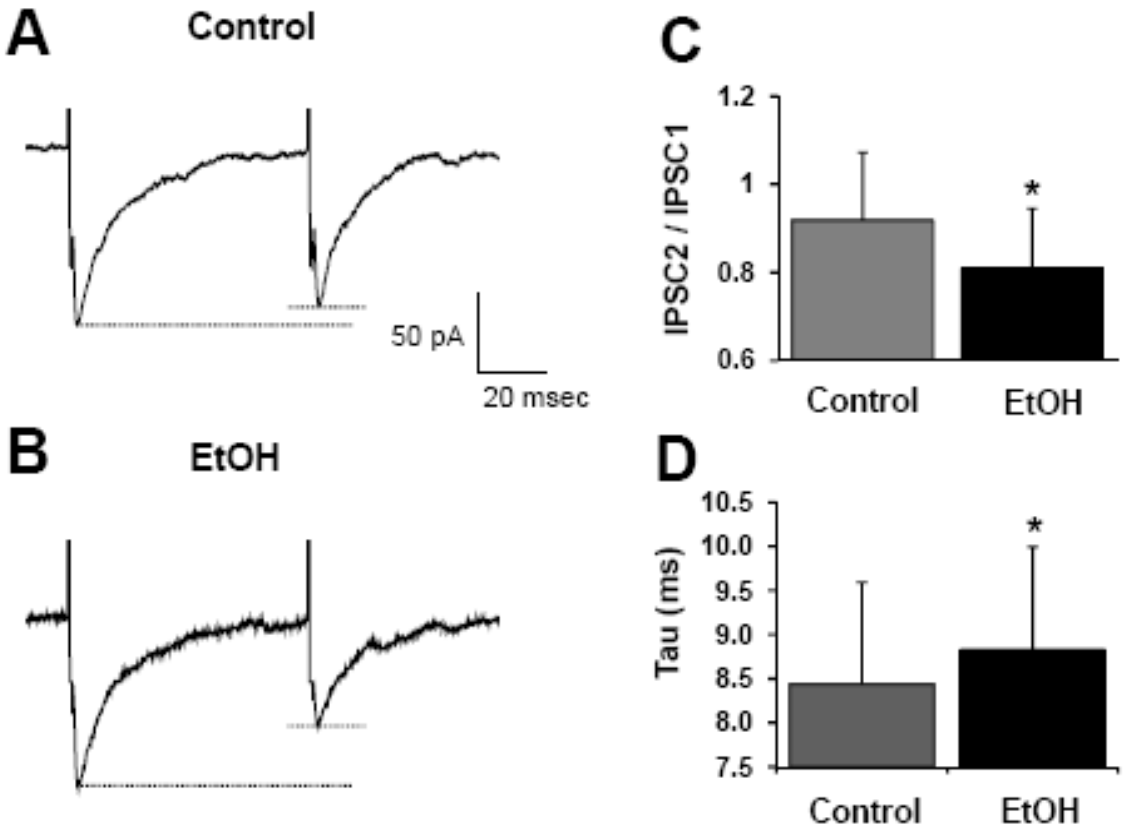


Figure 2-4. Ethanol enhances paired-pulse depression. A, B. Sample evoked IPSC recordings from a VTA-DA neuron prior to (A), and after exposure to ethanol (50 mM) (B). Each trace represents an averaged trace from 8 individual traces over a continuous 3.5 minute time period for each condition from a representative neuron. Both recordings exhibit paired-pulse depression (PPD), however after exposure to ethanol, the PPD is enhanced. C. A bar graph representing the paired-pulse ratio (defined as IPSC2/IPSC1) under control conditions and in the presence of ethanol (50 mM). D. A bar graph representing the decay time as measured by the time constant, τ (ms), under control conditions and in the presence of ethanol (50 mM). (n = 5 of 6, * indicates p < 0.05 by a paired student's t test different from control)

2-3-3. Ethanol potentiation of sIPSCs does not involve $GABA_B$ receptors

Activation of $GABA_B$ receptors on midbrain GABAergic interneurons inhibits GABA release from these neurons (Rohrbacher et al., 1997; Bonci and Malenka, 1999), and inhibition of $GABA_B$ receptors on hippocampal interneurons can increase GABA release (Mott and Lewis, 1991). Therefore, to test the possibility that ethanol may act via presynaptic $GABA_B$ receptors to increase GABA release, we recorded sIPSCs from VTA-DA neurons in the presence of either the $GABA_B$ agonist, baclofen, or the antagonist, SCH50911. In separate experiments, sIPSCs were recorded from VTA-DA neurons under control conditions followed by bath application of either baclofen (1.25 µM) or SCH50911 (20 µM), followed again by co-application of baclofen or SCH50911 with ethanol (50 mM). Application of baclofen alone reversibly decreased sIPSC frequency (Fig. 5A, B) but not amplitude (Fig. 5A, C). Subsequent co-application of ethanol and baclofen resulted in approximately a 30 percent increase in frequency above the baclofen-induced decrease with no significant change in amplitude. Application of SCH50911 (20 µM) significantly increased sIPSC frequency (Fig. 6A, B) with no significant change in amplitude (Fig. 6A, C) with respect to control. Subsequent co-application of ethanol and SCH50911 resulted in a 23 percent increase in frequency above SCH50911 alone that was reversible upon washout, with no apparent change in amplitude. Therefore, the results displayed in Figures 5 and 6 suggest that the ethanol-induced potentiation in sIPSC frequency is not affected by addition of a $GABA_B$ receptor agonist or antagonist.

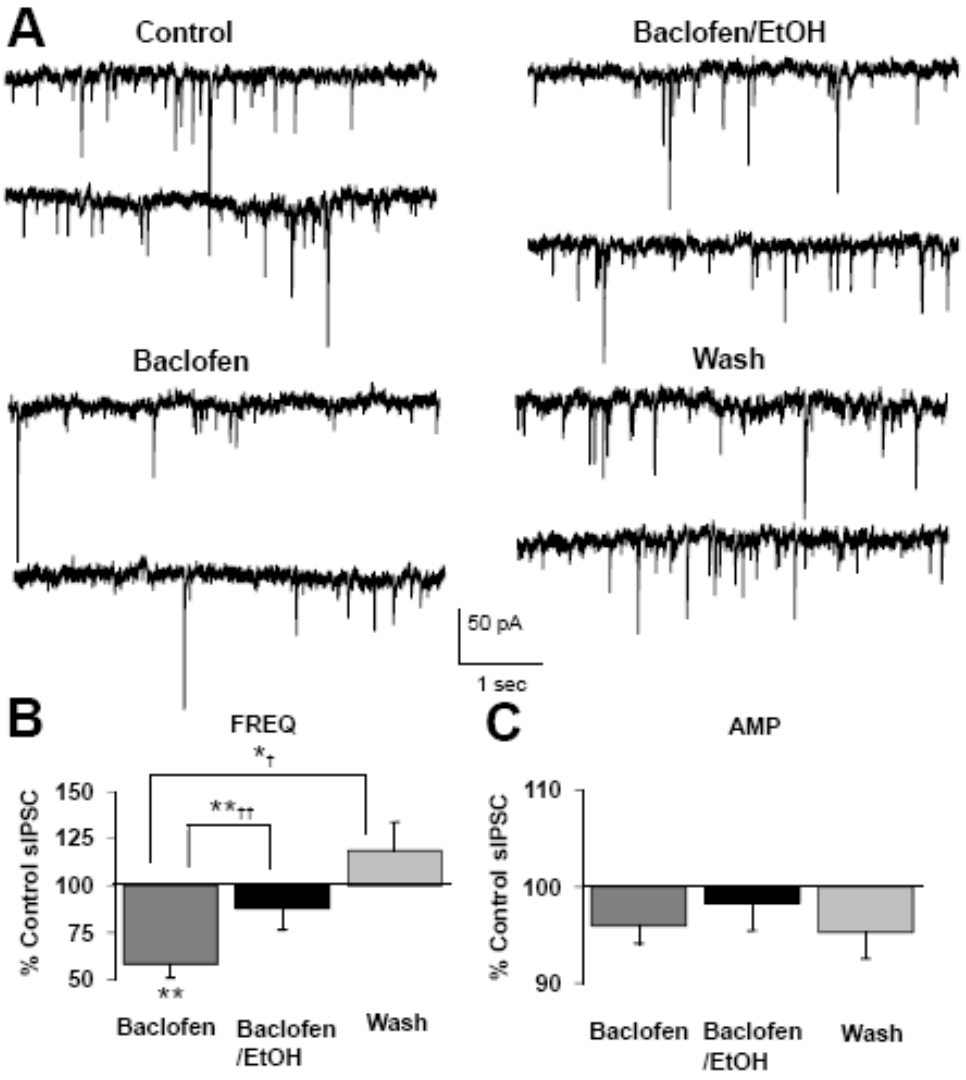


Figure 2-5. Baclofen does not inhibit ethanol-induced potentiation of sIPSC frequency. A. sIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 1.25 µM baclofen, 50 mM ethanol with 1.25 µM baclofen, and after a washout. B. A bar graph representing the % change ± SEM above control sIPSC frequency for the conditions shown in A. Event frequency under control conditions was 2.36 ± 0.23 Hz. C. A bar graph representing the % change ± SEM above control sIPSC amplitude for the conditions shown in A. Event amplitude under control conditions was 49.48 ± 3.41 pA. (n = 6, ** indicates $p < 0.01$ by ANOVA/Dunnett C post-hoc different from control, *† indicates $p < 0.05$ by ANOVA/Dunnett C post-hoc different from baclofen alone, **†† indicates $p < 0.01$ by a paired student's t test different from baclofen alone).

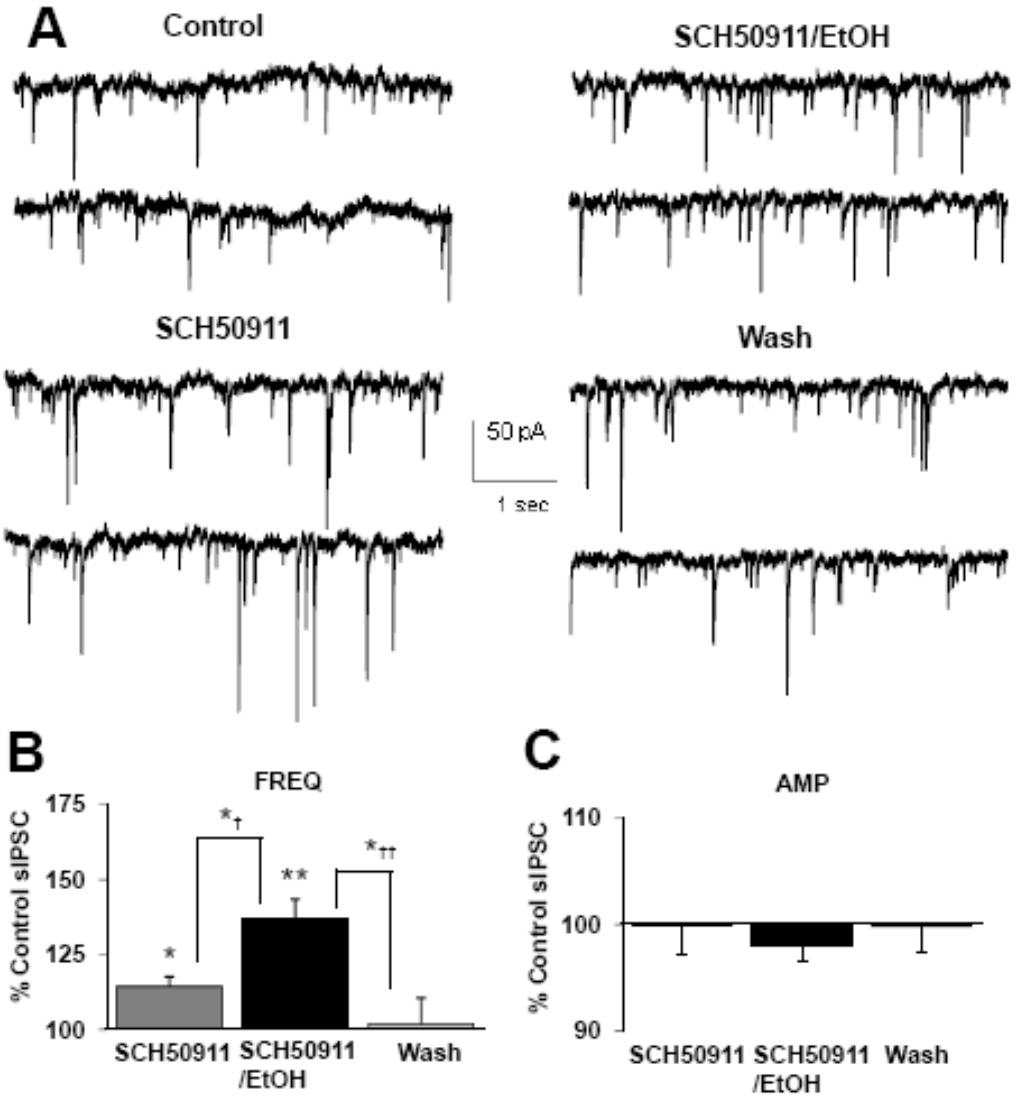


Figure 2-6. SCH50911 does not occlude ethanol-induced potentiation of sIPSC frequency. A. sIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 20 µM SCH50911, 50 mM ethanol with 20 µM SCH50911, and after a washout. B. A bar graph representing the % change ± SEM above control sIPSC frequency for the conditions shown in A. Event frequency under control conditions was 1.89 ± 0.40 Hz. C. A bar graph representing the % change ± SEM above control sIPSC amplitude for the conditions shown in A. Event amplitude under control conditions was 51.58 ± 2.24 pA. (n = 6, * indicates $p < 0.05$ by ANOVA/Tukey HSD post-hoc different from control, ** indicates $p < 0.01$ by ANOVA/Tukey HSD post-hoc different from control, *† indicates $p < 0.05$ by ANOVA/Tukey HSD post-hoc and by paired student's t test different from SCH50911 alone, *†† indicates $p < 0.05$ by ANOVA/Tukey HSD post-hoc different from wash).

2-3-4. Ethanol potentiates GABA release in the VTA

In order to further discern whether ethanol potentiates the frequency of spontaneous IPSCs by directly enhancing GABA release from presynaptic terminals or increasing the excitability of GABAergic interneurons, we recorded mIPSCs in the presence of TTX to block action potential-mediated release events. Application of ethanol (50 mM) produced a marked enhancement in mIPSC frequency (Fig. 7A, B), but not amplitude (Fig. 7A, C). A time course plot is shown in Fig. 7D to demonstrate the onset of ethanol action. These results support the notion that ethanol is acting presynaptically to enhance GABA release as evidenced by the sIPSC and eIPSC experiments.

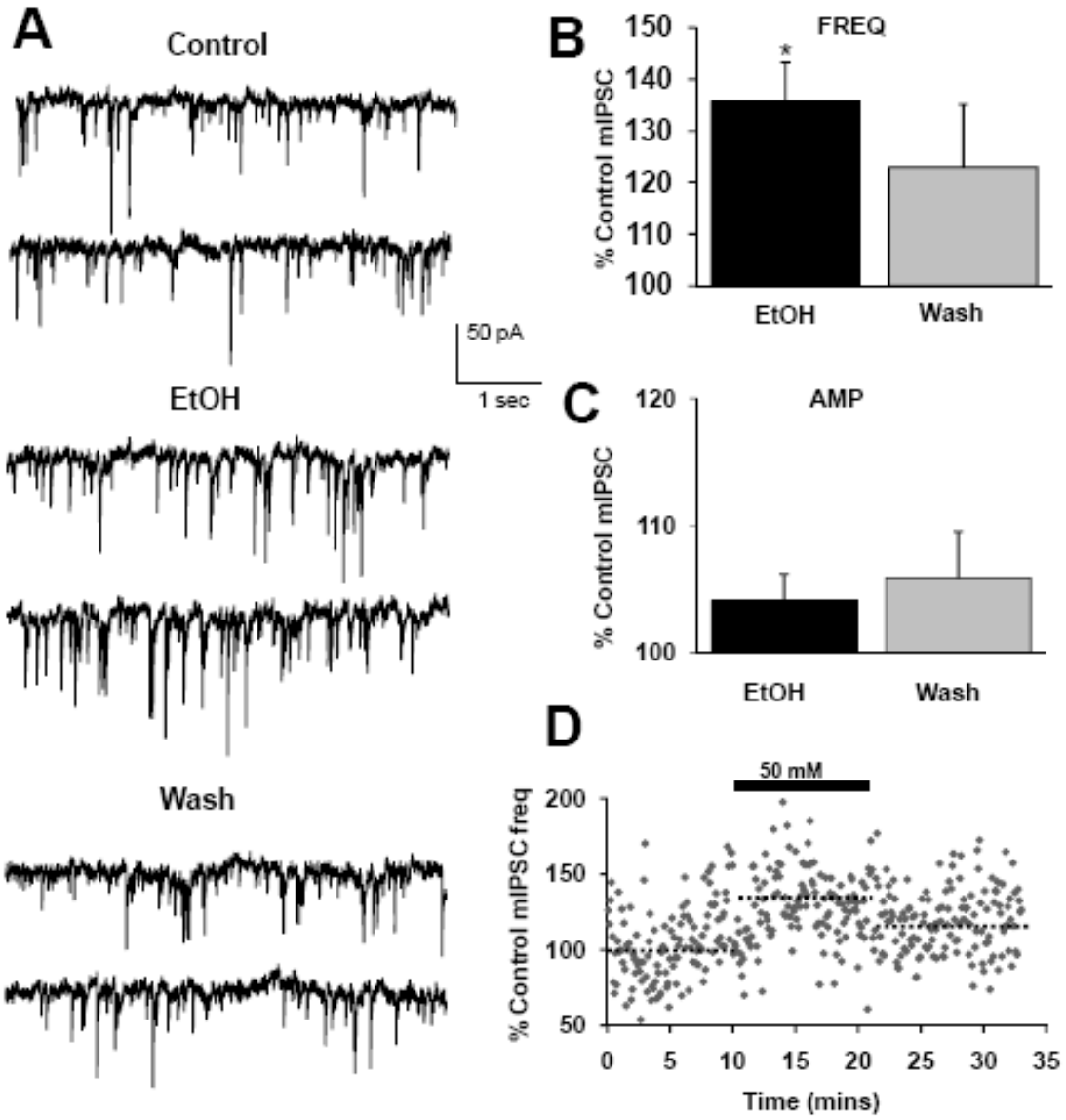


FIGURE 2-7. Ethanol (50 mM) potentiates mIPSC frequency. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 50 mM ethanol, and after a washout. B. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A. Event frequency under control conditions was 2.57 ± 0.45 Hz. C. A bar graph representing the % change ± SEM above control mIPSC amplitude for the conditions shown in A. Event amplitude under control conditions was 43.13 ± 3.88 pA. D. Cumulative time course for averaged cells. The dotted lines indicate the averaged value displayed in Fig. B. (n = 11, * indicates $p < 0.05$ by student's t test different from control).

2-4. Discussion

Our results demonstrate that ethanol enhances GABAergic transmission onto VTA-DA neurons in a concentration-dependent manner in brain slices, and that this effect is independent of presynaptic somatic/axonal excitability and $GABA_B$ receptor modulation. To our knowledge, this is the first study to demonstrate a direct and acute enhancement of GABAergic transmission by ethanol in the VTA. Several labs have reported both pre- and postsynaptic enhancement of GABAergic transmission in a variety of other cortical and subcortical brain regions in response to acutely administered ethanol. In rat cerebellar slices, acute ethanol increases the frequency of $GABA_A$-mediated sIPSCs recorded from granule cells (Carta et al., 2004). In the rat central amygdala (CeA), a brain region important in anxiety and stress responses, application of 44 mM ethanol facilitated GABAergic transmission via pre- and postsynaptic mechanisms (Roberto et al., 2003). Two recent reviews discuss the strong precedent for acute ethanol to enhance GABA release in the hippocampus, central amygdala, cerebellum, and nucleus accumbens (Siggins et al., 2005; Weiner and Valenzuela, 2006). Thus, a strong precedent for ethanol modulation of GABAergic transmission exists in a variety of CNS structures.

Other labs have demonstrated a reduction of GABAergic neuron excitability in the presence of ethanol in the VTA (Stobbs et al., 2004; Xiao et al., 2007); however neither lab explored GABA release specifically. It should be noted that a decrease in somatic excitability does not exclude a separate action

of ethanol to enhance terminal excitability. Ethanol inhibits NMDA receptors in VTA-GABAergic neurons (Stobbs et al., 2004) and this inhibition may partially explain the ethanol-induced reduction in GABAergic neuron excitability observed by Xial et al. Additionally, ethanol may increase GABA release by acting on terminals of GABAergic medium spiny neurons arising from the NAc and not on local VTA interneurons. Nonetheless, given the strong evidence of ethanol facilitation of GABA release in other CNS structures we are confident that our results demonstrate specific and direct effects on GABA release.

It is notable that in the concentration-response experiments in Figure 2, we typically did not observe a significant enhancement of GABAergic transmission after the initial exposure to 25 mM ethanol. However, that concentration of ethanol following a prior exposure to 15 mM ethanol did result in significant enhancement of sIPSC frequency but not amplitude. One possible explanation of this effect of successive applications of ethanol is that such intermediate concentrations of ethanol may elicit slowly developing alterations in GABAergic transmission and such a time course of slow onset may provide clues about potential mechanisms. On the other hand, an alternative explanation is that the lower ethanol concentrations may sensitize synapses to subsequent ethanol applications. Furthermore, it is important to note that we are using young rats (21-30 days old) in our experiments. In a study comparing the effect of ethanol on sIPSCs and mIPSCs between juvenile and adult rats, ethanol had a greater effect on sIPSCs in adults than juveniles (Li et al., 2006). However, there

were no differences in mIPSCs between the adult and juvenile rats. Therefore, it is possible that the variability seen in our sIPSC recordings with these intermediate ethanol concentrations may reflect the slight variability in the ages of the rats used within each group of experiments.

The increase in sIPSC frequency in the presence of ethanol may indicate an increase in terminal GABA release probability or an increase in interneuron somatic/axonal excitability. To discern the former from the latter, we measured the paired-pulse ratio of evoked IPSCs and the frequency of mIPSCs recorded in the presence of TTX. Under control conditions we observed paired-pulse depression; however, acutely applied ethanol further amplified the depression, suggesting an increased probability of GABA release from presynaptic terminals. Additionally, we saw a robust and reliable increase in mIPSC frequency in the presence of ethanol, also suggestive of enhanced GABA release independent of interneuron somatic/axonal excitability. We did not observe a change in mIPSC amplitude in the presence of ethanol. These findings are consistent with other reports in which ethanol increases mIPSC frequency while having no effect on postsynaptic $GABA_A$ receptor sensitivity (Li et al., 2006; Ming et al., 2006; Zhu and Lovinger, 2006; Kelm et al., 2007). Although we did observe variable changes in sIPSC amplitude in the presence of ethanol, the lack of change in postsynaptic sensitivity with mIPSCs may be a result of the elimination of larger action-potential induced events by TTX. Taken together, our electrophysiological

recordings therefore strongly indicate that ethanol positively modulates GABA release onto VTA-DA neurons.

$GABA_B$ receptor activation has been implicated in some actions of ethanol (Ariwodola and Weiner, 2004) and therefore we assessed the potential involvement of $GABA_B$ receptors in ethanol modulation of GABA transmission. We observed a decrease in sIPSC frequency mediated by the $GABA_B$ receptor agonist, baclofen, alone which confirmed previous findings (Rohrbacher et al., 1997). Conversely, we noted an unexpected increase in frequency following application of the antagonist SCH50911 alone which suggests that tonic GABA release elicits tonic $GABA_B$ receptor-mediated auto-inhibition of GABAergic neurons. Application of the $GABA_B$ receptor antagonist presumably removes this tonic auto-inhibition, allowing for increased GABA release. Nevertheless, neither activation nor blockade of $GABA_B$ receptors appeared to occlude the ethanol enhancement in sIPSC frequency indicating ethanol modulation of GABA release is independent of $GABA_B$ receptors. Interestingly, application of either the $GABA_B$ receptor agonist or antagonist completely blocked the enhancement in amplitude. The reason as to the occlusion of the postsynaptic effect is not clear, however, there is evidence of $GABA_B$ receptor modulation affecting postsynaptic $GABA_A$ receptor ethanol sensitivity (Wu et al., 2005). Nevertheless, the initial degree of sIPSC amplitude enhancement seen with ethanol (50 mM), while significant, is less than 10% over the control sIPSC amplitude value and this effect did not reverse upon ethanol washout. Thus, while we report these small

changes in amplitude, we do not feel these constitute convincing evidence to indicate that ethanol modulation of sIPSC amplitude is nearly as significant as its effects on m/sIPSC frequency and thus its modulation of GABA release. In this regard, we can therefore unequivocally state that the robust presynaptic actions of ethanol are not modulated in any manner by activation or inhibition of $GABA_B$ receptors.

Multiple labs (including our own – data not shown) have demonstrated an enhancement in VTA-DA neuron firing rate and increased dopamine release into the NAc after exposure to ethanol and this action is thought to underlie the addictive nature of this drug (Brodie et al., 1990; Gonzales and Weiss, 1998; Kohl et al., 1998; Brodie et al., 1999b; Okamoto et al., 2006). Our present observations of ethanol enhancement of GABAergic transmission therefore would appear to antagonize such stimulatory actions especially since GABAergic interneurons regulate DA neuron excitability (Johnson and North, 1992a). Furthermore, VTA-DA neurons undergo tonic modulation by GABAergic interneurons since prior studies have shown that $GABA_A$ receptor blockade increases extracellular dopamine levels in the NAc (Ikemoto et al., 1997). Thus, the potentiation of DA neuron excitability by ethanol may be self-limiting due to the simultaneous ethanol-induced enhancement in inhibitory drive onto those same neurons. Therefore, there potentially exists a biphasic action of ethanol first to increase VTA-DA excitability directly and secondly to enhance GABAergic inhibition of VTA-DA neurons. This inhibitory action of ethanol may in effect

constitute an additional pharmacologic action of significant importance that results in a balance of inhibitory versus excitatory effects to regulate DA neuron firing rate and therefore may constitute an important target for further evaluation. A similar effect has recently been observed in cerebellar Purkinje neurons in which ethanol has dual actions on these neurons to increase GABA release presynaptically while simultaneously acting postsynaptically to increase cell firing rate (Ming et al., 2006).

While the increase in GABA release by ethanol is insufficient to prevent the excitatory effect(s) of ethanol on VTA-DA neurons, whether there exists a subset of DA neurons in the VTA that might display differential sensitivity to the excitatory and inhibitory effects of ethanol remains to be determined. This is quite possible considering a recent study that discerned two populations of VTA-DA neurons with differential sensitivity to opioid-mediated presynaptic inhibition (Ford et al., 2006). Nevertheless, our data clearly demonstrate that ethanol enhances GABA release in the VTA. The mechanism(s) underlying this action are currently under investigation and preliminary results suggest one potential route may involve ethanol enhancement of presynaptic Ca^{2+} mobilization via a 5-HT_{2C}-dependent mechanism. Since ethanol modulation of ventral tegmental DA output function is critically involved in reinforcement and reward, identification of such regulatory pathways are critical to construct an accurate picture of the neuropharmacological actions of ethanol.

Chapter 3: Role of 5-Hydroxytryptamine$_{2C}$ Receptors in Ca^{2+}-dependent Ethanol Potentiation of GABA Release onto Ventral Tegmental Area Dopamine Neurons

 Theile JT, Morikawa H, Gonzales RA, Morrisett RA. Role of 5-Hydroxytryptamine$_{2C}$ Receptors in Ca^{2+}-dependent Ethanol Potentiation of GABA Release onto Ventral Tegmental Area Dopamine Neurons. JPET 329:625–633, 2009.

Activation of ventral tegmental area (VTA) dopaminergic (DA) neurons by ethanol has been implicated in the rewarding and reinforcing actions of ethanol. GABAergic transmission is thought to play an important role in regulating the activity of DA neurons. We have previously reported that ethanol enhances GABA release onto VTA-DA neurons in a brain slice preparation. Since intra-terminal Ca^{2+} levels regulate neurotransmitter release, we investigated the roles of Ca^{2+}-dependent mechanisms in ethanol-induced enhancement of GABA release. Acute ethanol enhanced miniature IPSC (mIPSC) frequency in the presence of the nonspecific voltage-gated Ca^{2+} channel inhibitor, cadmium chloride, even though basal mIPSC frequency was reduced by cadmium. Conversely, the IP_3 receptor inhibitor, 2-APB, and the sarco/endoplasmic

reticulum Ca^{2+} ATPase pump inhibitor, cyclopiazonic acid, eliminated the ethanol-enhancement of mIPSC frequency. Recent studies suggest that the G protein-coupled receptor, 5-HT_{2C}, may modulate GABA release in the VTA. Thus, we also investigated the role of 5-HT_{2C} receptors in ethanol-enhancement of GABAergic transmission. Application of 5-HT and the 5-HT_{2C} receptor agonist, Ro-60-0175, alone enhanced mIPSC frequency of which the latter was abolished by the 5-HT_{2C} receptor antagonist, SB200646, and substantially diminished by cyclopiazonic acid. Furthermore, SB200646 abolished the ethanol-induced increase in mIPSC frequency while having no effect on basal mIPSC frequency. These observations suggest that an increase in Ca^{2+} release from intracellular stores via 5-HT_{2C} receptor activation is involved in the ethanol-induced enhancement of GABA release onto VTA-DA neurons.

3-1. Introduction

The mesolimbic system bidirectionally encodes information related to positively- and negatively-reinforcing stimuli, and with conditioning, will adapt its output to such stimuli in a manner which encodes the error in reward prediction (Schultz et al., 1997). This capability is primarily processed via ventral tegmental area dopaminergic neurons (VTA-DA) which originate in the midbrain nucleus A10 and project to the nucleus accumbens (NAc), prefrontal cortex, basolateral amygdala and other corticolimbic structures (Albanese and Minciacchi, 1983; Oades and Halliday, 1987). Natural positive-reinforcers activate VTA-DA neurons to release DA onto these structures, of which the medium spiny neurons

of the NAc constitute the primary VTA-DA target. Most drugs of abuse pharmacologically activate VTA-DA neurons via a variety of mechanisms culminating in aberrant release of DA onto these targets and such pathological activation of the mesolimbic pathway is considered a primary step in the development and expression of drug dependence and addiction. Thus, acute exposure to ethanol increases dopamine levels in the NAc (Weiss et al., 1993). Furthermore, chronic exposure to ethanol has been reported to downregulate VTA-DA release and this state of dopaminergic hypofunction is thought to contribute to alcohol craving in dependent individuals (Rossetti et al., 1992). While the mechanisms by which ethanol activates VTA-DA neurons to enhance DA output to the NAc are not entirely clear, evidence suggests a direct excitatory effect of ethanol on DA neurons in the VTA either though inhibition of delayed-rectifying K^+ channels or modulation of the h-current which underlies the basal pacemaker function of VTA neurons (Gessa et al., 1985; Brodie et al., 1999b; Okamoto et al., 2006; Koyama et al., 2007).

Dopamine output from the VTA is regulated by both local GABAergic interneurons and GABA containing afferents from the NAc and ventral pallidum (Walaas and Fonnum, 1980; Grace and Onn, 1989; Johnson and North, 1992a). Remarkably, our understanding of the effects of ethanol on GABAergic transmission in the mesolimbic DA system is not well defined and is currently in dispute. We have reported that pharmacologically relevant concentrations of ethanol enhance action potential-dependent and -independent GABA release

onto VTA-DA neurons (Theile et al., 2008). On the other hand, Ye and colleagues recently demonstrated that ethanol decreases action-potential dependent GABA release onto VTA-DA neurons in a manner reversed by the mu opioid receptor agonist, DAMGO (Xiao and Ye, 2008). This discrepancy is particularly important since one of the few currently FDA-approved agents for alcohol craving - the mu opiate antagonist, naltrexone - is thought to directly modulate VTA-GABAergic neuron activity and thus indirectly affect VTA-DA neuron firing (Franklin, 1995). Indeed, naltrexone has been demonstrated to block the ethanol-induced increase in NAc dopamine levels in animal models (Gonzales and Weiss, 1998). Thus, in this report we have focused our work to uncover VTA-GABAergic regulatory mechanisms which may indirectly modulate ethanol effects on VTA-DA output.

One candidate regulatory pathway to the VTA consists of serotoninergic afferents from the midbrain raphe nuclei which innervate both DA and non-DA (GABAergic) neurons of the VTA (Herve et al., 1987). Electrophysiological evidence indicates that serotonin (5-hydroxytryptamine; 5-HT) reuptake blockade inhibits VTA-DA firing suggesting that a predominant action of 5-HT in the VTA is inhibitory and may involve activation of GABAergic neurons (Di Mascio et al., 1998). This contention is supported by the observations that (1) 5-HT_{2C} receptor (5-HT_{2C}R) activation also inhibits VTA-DA neuron firing (Di Matteo et al., 2000), (2) 5-HT_{2C}R mRNA is expressed in GABAergic neurons (Eberle-Wang et al., 1997) and (3) 5-HT_{2C}Rs are localized to both synaptic terminals and cell soma of

VTA-GABA neurons though these receptors have also been reported to occur on DA neurons as well (Bubar and Cunningham, 2007). Since 5-HT_{2C}Rs facilitate phospholipase C-mediated inositol-1,4,5- triphosphate (IP_3) accumulation and release of Ca^{2+} from intracellular stores (Conn et al., 1986), activation of this pathway on GABAergic soma or terminals could very well result in enhancement of GABA release. Indeed, activation or inhibition of 5-HT_{2C}Rs decreases or increases respectively, ethanol self-administration by rats (Tomkins et al., 2002). Also, enhancement of GABA release by ethanol onto cerebellar Purkinje neurons is dependent upon mobilization of Ca^{2+} from intracellular stores (Kelm et al., 2007). Lastly, no prior reports exist which directly assess whether 5-HT_{2C}R activation regulates GABA release onto VTA-DA neurons. Therefore, another major impetus for this study is to identify the role of 5-HT_{2C}Rs and subsequent intracellular Ca^{2+} mobilization in ethanol facilitation of GABA release onto VTA-DA neurons.

3-2. Materials and Methods

3-2-1. Slice preparation

All experiments were carried out in accordance with NIH animal use guidelines and were approved by the University of Texas Institutional Animal Care and Use Committee. Slices used in this study were prepared from male Sprague-Dawley rats (postnatal day 21 to 33). Rats were anesthetized with halothane, decapitated, and the brain was rapidly removed and placed in an ice-cold choline-based, oxygenated artificial cerebrospinal fluid (aCSF) containing (in

mM): 110 choline Cl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 dextrose, 7 $MgSO_4$, 0.5 $CaCl_2$, 11.6 Na-ascorbate and 3.1 Na-pyruvate, bubbled with 95% O_2/5% CO_2 (all chemicals obtained from Sigma-Aldrich, St Louis, MO). Horizontal midbrain slices (210 µm) were prepared using a vibrating slicer (VT1000S; Leica, Nussloch, Germany). The slices were then maintained at 32^{o}C before electrophysiological recordings for a minimum of 60 minutes in aCSF containing (in mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KCl, 1.23 NaH_2PO_4, 10 dextrose, 2.4 $MgSO_4$, and 1.8 $CaCl_2$, bubbled with 95%O_2/5%CO_2.

3-2-2. Electrophysiological recordings of VTA-DA neurons

Individual slices were transferred to a recording chamber and perfused with oxygenated aCSF (30-32^{o}C) at a flow rate of ~2 ml/min. Recording aCSF was as described above except it contained 0.9 mM $MgSO_4$ and 2 mM $CaCl_2$. Cells were visualized using IR-DIC optics on an Olympus BX-50WI microscope (Leeds Instruments, Irving, TX). The VTA was identified as being medial to the medial terminal nucleus of the accessory optic tract (MT) and rostral to the oculomotor nerve (3n) and the medial lemniscus (ml). The majority of recordings were conducted in the lateral VTA, just medial to the MT. Whole-cell voltage-clamp recordings were used for all experiments; putative DA neurons were identified by the presence of a large hyperpolarization-induced I_h current (>200 pA) that was measured immediately following break-in by application of a 1.5-s hyperpolarizing step from -60 to -110 mV (Johnson and North, 1992b). Recording electrodes were made from thin-walled borosilicate glass (TW 150F-4,

WPI, Sarasota, Florida, 1.5-2.5 MΩ) and contained (in mM): 135 KCl, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Tris-GTP, pH 7.3 with KOH (all chemicals obtained from Sigma, St Louis, MO). Data were collected by an Axon Instruments Model 200B amplifier filtered at 1 kHz and digitized at 10-20 kHz with a Digidata interface using pClamp v9.2 and 10.2 (Molecular Devices, Sunnyvale, California).

GABAergic mIPSCs were pharmacologically isolated with kynurenic acid (1 mM) to inhibit AMPA- and NMDA receptor-mediated currents. Tetrodotoxin (TTX; 0.5 µM) and eticlopride (250 nM) were included to block Na^+ currents and D_2 receptor-mediated currents, respectively. Under these conditions, mIPSCs were inward at a holding potential of -60 mV and in an initial set of experiments their identity as GABAeric events was verified by testing for block with picrotoxin or bicuculline (data not shown). Following break-in and a stable 10-minute baseline (control) recording, drugs were bath-applied through the aCSF perfusion line and a continuous 10-15 minute recording epoch was used to detect changes in mIPSC frequency and amplitude. A four-minute drug wash-on preceded the start of data collection in each treatment condition, and a 12-min washout period followed drug application. The last half (6 minutes) of the washout period was used in our data analysis. The number of neurons used per each treatment condition is represented as *n* with only one neuron used per slice. Eticlopride hydrochloride, 2-APB, cyclopiazonic acid, bicuculline methiodide and Ro-60-0175 fumarate were obtained from Tocris Bioscience (Ellisville, MO). Kynurenic acid,

SB200646, nicardipine hydrochloride, cadmium chloride, serotonin hydrochloride and picrotoxin were obtained from Sigma-Aldrich (St. Louis, MO). TTX was obtained from Alomone Labs (Jerusalem, Israel).

3-2-3. Data analysis

For mIPSC recordings, quantal events (90-120 sweeps each condition, 5 sec/sweep) were detected using the template mIPSC detection protocol contained within pClampfit (pClamp v9.2, Axon Inst., Molecular Devices). Access resistance ranged from 6 to 20 MΩ and was monitored throughout the experiment. Experiments where access resistance changed (>20% at anytime during the experiment) were excluded from this study. To minimize detecting small noise deflections as mIPSCs (false positives), events <10 pA were discarded. Treatment and washout groups were normalized to the baseline (control) frequency or amplitude and represented as a percentage of the control. Averaged values for all data sets are expressed as mean ± SEM and were compared statistically using paired student's *t* test, unpaired student's *t* test, one-way analysis of variance (ANOVA) and Bonferroni post hoc test where mentioned. The events encompassed in the histogram insets (Fig. 1) were compared using a Kolmogorov-Smirnov (K-S) test. Significant differences were considered as $*p < 0.05$ and $**p < 0.01$.

3-3. Results

3-3-1. Ethanol enhances mIPSC frequency

Acute ethanol exposure enhanced GABA release onto VTA-DA neurons recorded in an in vitro slice preparation (Theile et al., 2008). For studies on the ethanol concentration-dependence of GABA mIPSCs, a total of 32 VTA-DA neurons were exposed to 15, 25, 50 or 75 mM ethanol. All concentrations displayed an increase in mIPSC frequency (Fig 1D) while no consistent change in mIPSC amplitude was apparent at any ethanol concentration (data not shown). The enhancement in frequency by both 50 and 75 mM were significant compared to control ($p < 0.05$, one-way ANOVA, Bonferroni post-hoc). There was an apparent increase in mIPSC frequency at 15 mM similar to that seen with 25 mM, however, the change was not significant. In response to 25 mM ethanol (legal intoxication is 17.4 mM), the frequency of mIPSCs increased by approximately 20%; mIPSC frequency increased by about 30 and 50% following exposure to 50 and 75 mM ethanol, respectively. In order to more thoroughly define the actions of ethanol on GABA mIPSC frequency and amplitude, we studied the cumulative event distributions for sample neurons at all concentrations tested (only 75 mM ethanol shown; Fig 1B/C). Application of ethanol induced a left-ward shift in the cumulative distributions of mIPSC frequency (measured as inter-event interval) which were statistically significant at all concentrations tested (K-S test, $p < 0.05$). The shifts in mIPSC frequency distributions were not skewed across different frequency ranges and thus were independent of mIPSC frequency itself. The

insets for Fig 1B/C presents an expanded section of the event distributions and is centered around the 70-90th percentile level for more detailed comparison between treatments and groups. In all cases, shifts in mIPSC frequency are apparent and are evenly distributed across frequencies whereas no changes in amplitude distributions are apparent. Finally, mIPSC frequency washout data reflects a notable shift back toward the control (pre-ethanol) population distribution.

To ensure that changes in postsynaptic or extra-synaptic GABA receptor function were not overlooked, we also measured the effects of ethanol on the decay time constant, τ, of the mIPSCs as well as the baseline holding current as a measure of tonic extra-synaptic GABA receptor function (data not shown). At the highest concentration tested (75 mM) ethanol did not affect decay kinetics, or tau (τ), of the mIPSC. Under control conditions, mIPSC decay was 6.8±0.6 ms, whereas in the presence of ethanol it was 6.7±0.5 ms (n = 7, p = 0.66). We additionally found no significant effect of ethanol (75 mM) to enhance or induce tonic steady-state or "holding" currents recorded from VTA-DA neurons at -60 mV. For instance, a sham perfusion buffer exchange resulted in a positive shift in the average holding current of 24±10 pA (n = 7), whereas ethanol treatment increased the average holding current by a similar degree (10 ±10 pA (n =7)).

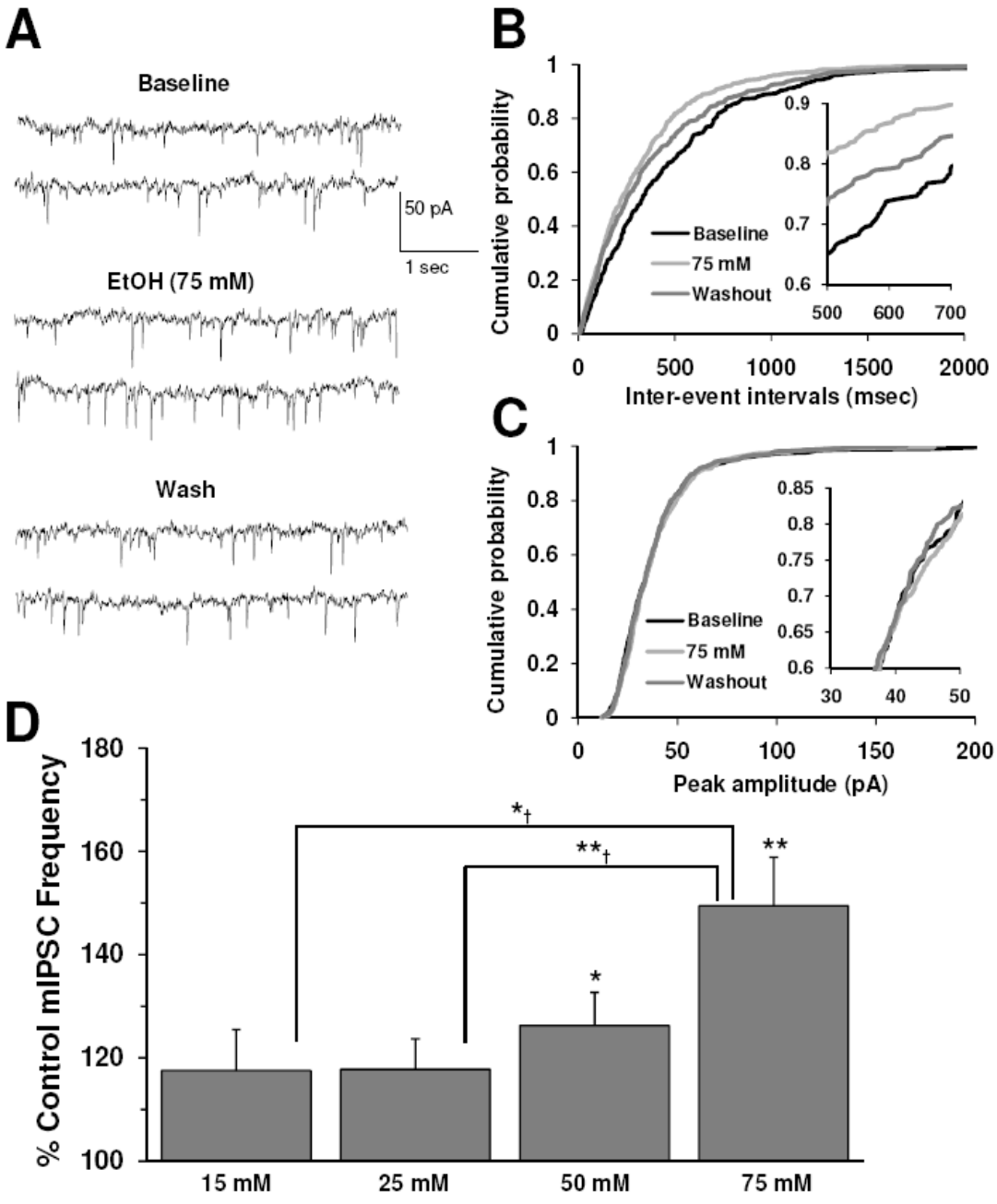

A
Baseline
50 pA
1 sec
EtOH (75 mM)
Wash
B
Cumulative probability
Baseline
75 mM
Washout
Inter-event intervals (msec)
C
Cumulative probability
Baseline
75 mM
Washout
Peak amplitude (pA)
D
% Control mIPSC Frequency
15 mM
25 mM
50 mM
75 mM

Figure 3-1. Ethanol potentiates mIPSC frequency but not amplitude. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 75 mM ethanol, and after a washout. B. Cumulative probability histogram of mIPSC inter-event intervals for epochs from a representative neuron. *Inset*: The leftward shift in IEI distribution in the presence of ethanol is significant with respect to baseline (K-S test, $p < 0.05$). C. Cumulative probability histogram of mIPSC event amplitudes from the same neuron shown in B. *Inset*: There is no shift in amplitude distribution in the presence of ethanol with respect to baseline (K-S test, $p > 0.05$). D. A bar graph representing the % change $\pm$ SEM above control mIPSC frequency at 15, 25, 50, and 75 mM ethanol ($n = 7$ for 15 mM; $n = 8$ for 25 mM; $n = 10$ for 50 mM; $n = 7$ for 75 mM; * indicates $p < 0.05$ different from control, ** indicates $p < 0.01$ different from control, *† indicates $p < 0.05$ different from 15 mM and **† indicates $p < 0.01$ different from 25 mM by one-way ANOVA, Bonferroni post hoc test).

3-3-2. Extracellular Ca^{2+} influx is not required for the ethanol-induced increase in GABA release

To determine whether Ca^{2+} influx from extracellular sites may contribute to ethanol-induced enhancement of GABA release, we examined the effects of the nonselective voltage-gated Ca^{2+} channel (VGCC) inhibitor, cadmium chloride ($CdCl_2$; 100 μM) on ethanol potentiation of mIPSC frequency (Fig. 2A, B). In the presence of $CdCl_2$ alone, mIPSC frequency markedly decreased indicating that influx of Ca^{2+} may significantly contribute to basal quantal GABA release even when action potentials were blocked with TTX. There was also a slight decrease in mIPSC amplitude (91.6 $\pm$ 2.2% of control; data not shown) in the presence of $CdCl_2$ that remained in the presence of ethanol. Nevertheless, subsequent application of ethanol (50 mM) in the continued presence of $CdCl_2$ significantly increased mIPSC frequency and therefore reversed the apparent $CdCl_2$ inhibition by about 27%. Following removal of both ethanol and $CdCl_2$ from the perfusate, mIPSC frequency reversed to levels substantially greater than the pre-$CdCl_2$ baseline indicating that prolonged inhibition of extracellular Ca^{2+} availability may induce a complex rebound phenomenon which appears to markedly affect GABA release.

Of the different types of VGCCs, interactions with the L-type VGCC subtype and ethanol have been frequently noted (Mullikin-Kilpatrick and Treistman, 1995; Hendricson et al., 2003). GABA release onto the cholinergic neurons of Meynert's nucleus has been shown to be dependent upon L-type

channels (Rhee et al., 1999). Therefore, we also assessed the effects of the L-type VGCC antagonist, nicardipine (3 μM) on ethanol potentiation of mIPSC frequency (Fig. 2C,D). Application of this VGCC antagonist resulted in no change in either baseline mIPSC frequency (Fig. 2D) or amplitude (data not shown). As observed with $CdCl_2$, in the continued presence of nicardipine, subsequent application of ethanol (50 mM) enhanced mIPSC frequency by 40$\pm$13% and slightly increased mISPC amplitude by approximately 8% (control: 32.1 $\pm$ 2.2 pA, n = 6, $p < 0.05$). Taken together, these results suggest that extracellular Ca^{2+} influx, presumably via N- and/or P/Q-type VGCCs, contribute to basal GABA release, whereas L-types do not. Nevertheless, under no condition was ethanol-enhancement of GABA release abolished by blocking extracellular Ca^{2+} entry.

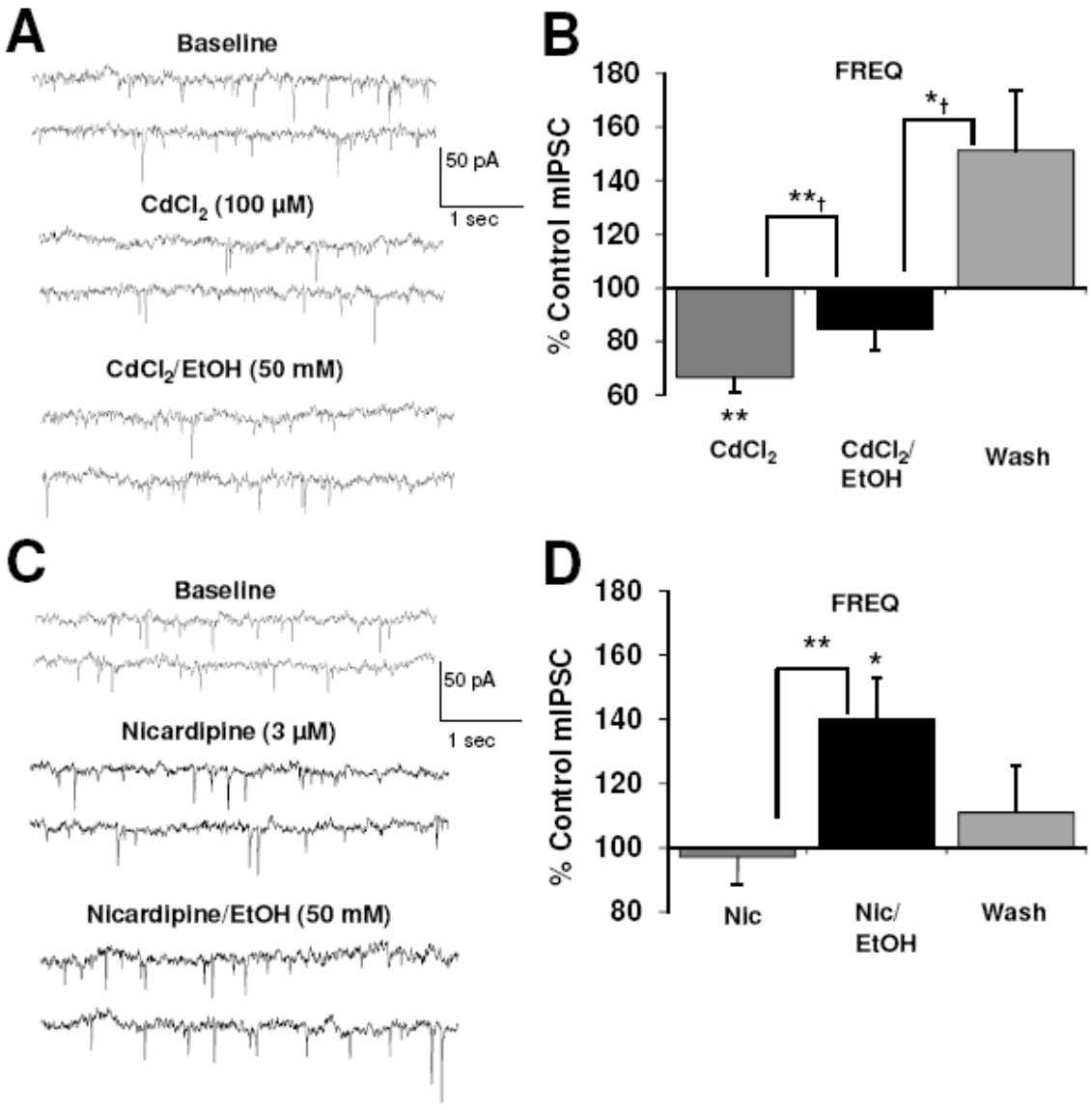

A
Baseline
50 pA
1 sec
CdCl2 (100 µM)
CdCl2/EtOH (50 mM)
B
FREQ
% Control mIPSC
180
160
140
120
100
80
60
**†
*†
**
CdCl2
CdCl2/ EtOH
Wash
C
Baseline
50 pA
1 sec
Nicardipine (3 µM)
Nicardipine/EtOH (50 mM)
D
FREQ
% Control mIPSC
180
160
140
120
100
80
**
*
Nic
Nic/ EtOH
Wash

Figure 3-2. Blockade of VGCCs does not inhibit ethanol-induced potentiation of mIPSC frequency. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 100 µM $CdCl_2$, and 50 mM ethanol with 100 µM $CdCl_2$. B. A bar graph representing the % change $\pm$ SEM above control mIPSC frequency for the conditions shown in A and a wash. Event frequency under control conditions was 1.7 $\pm$ 0.3 Hz (n = 10, ** indicates $p < 0.01$ by a student's t test different from control, *† indicates $p < 0.05$ by a student's t test different from $CdCl_2$/EtOH, **† indicates $p < 0.01$ by a paired student's t test different from $CdCl_2$ alone). C. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 3 µM nicardipine, and 50 mM ethanol with 3 µM nicardipine. D. A bar graph representing the % change $\pm$ SEM above control mIPSC frequency for the conditions shown in C and a wash. Event frequency under control conditions was 2.0 $\pm$ 0.4 Hz (n = 6, * indicates $p < 0.05$ by a student's t test different from control, ** indicates $p < 0.01$ by a paired student's t test different from nicardipine alone).

3-3-3. Intracellular Ca^{2+} stores are required for ethanol-induced enhancement of GABA release

Ca^{2+} release from presynaptic IP_3- and ryanodine –sensitive internal stores is necessary for ethanol potentiation of GABA release onto Purkinje neurons in the cerebellum (Kelm et al., 2007). Thus we investigated the role of IP_3-sensitive intracellular Ca^{2+} in ethanol enhancement of mIPSC frequency through the use of two antagonists. First we assessed the actions of the IP_3 receptor (IP_3R) antagonist, 2-Aminoethoxydiphenylborane (2-APB), and secondly, we studied an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, cyclopiazonic acid (CPA) which depletes intracellular Ca^{2+} stores (Seidler et al., 1989). As also noted by Kelm et al (2007) in their studies on Purkinje neurons, application of 2-APB alone (Fig. 3A, B) had no significant effect on mIPSC frequency or amplitude. However, subsequent addition of ethanol in the presence of 2-APB did not result in any further change in frequency or amplitude as compared to 2-APB alone. Likewise, application of the SERCA pump inhibitor, CPA (10 µM), at a concentration shown to be effective in depleting intracellular Ca^{2+} stores in DA neurons (Morikawa et al., 2000), completely blocked the enhancement of mIPSC frequency induced by subsequent addition of ethanol (50 mM) (Fig. 3C,D). CPA alone did not change mIPSC frequency or amplitude compared to baseline. These results suggest that IP_3R-mediated release of Ca^{2+} from intracellular stores is required for ethanol enhancement of GABA release onto DA neurons.

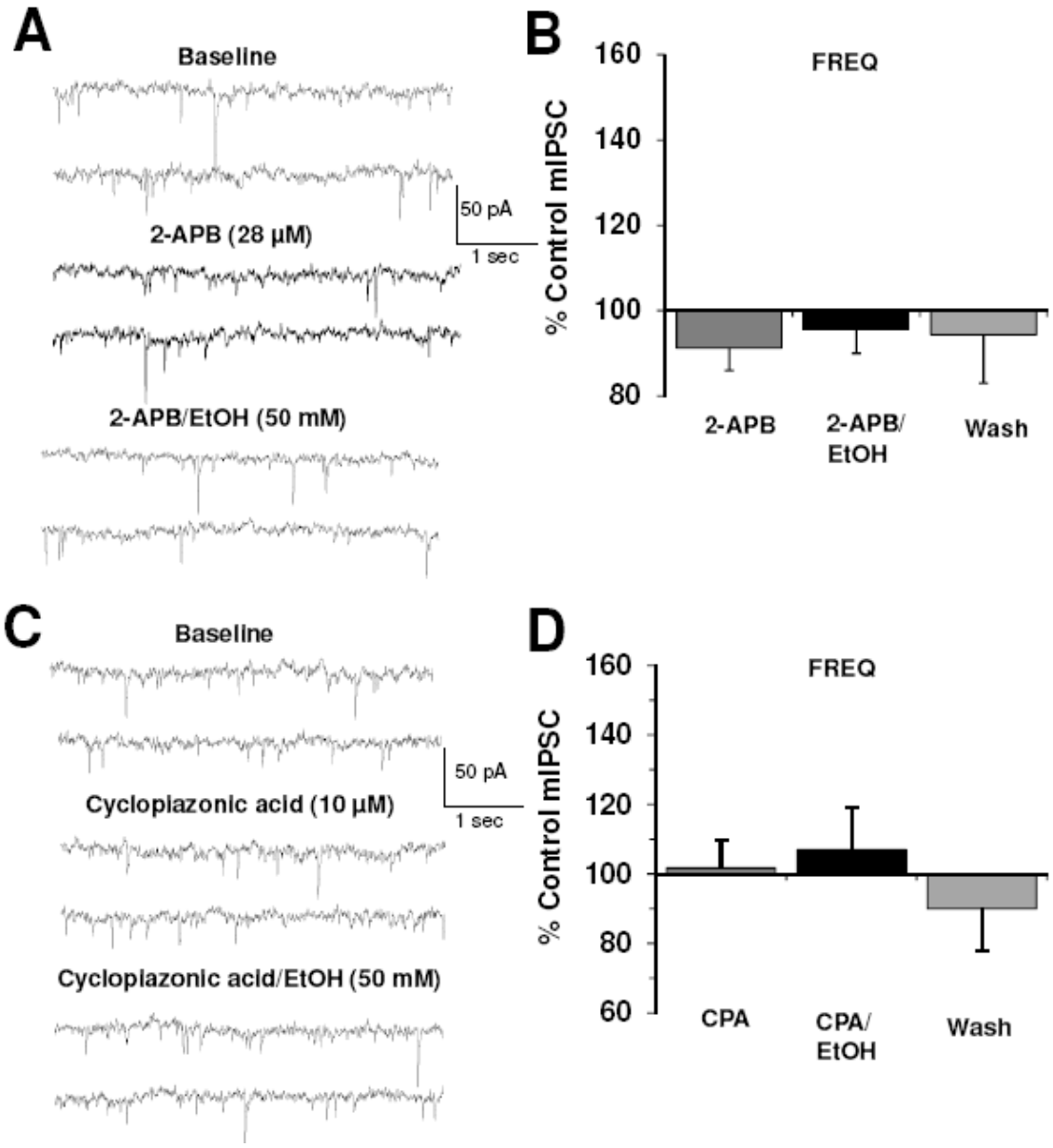


Figure 3-3. Blockade of intracellular Ca^{2+} release abolishes ethanol-induced potentiation of mIPSC frequency. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 28 µM 2-APB, and 50 mM ethanol with 28 µM 2-APB. B. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A and a wash. Event frequency under control conditions was 2.1 ± 0.6 Hz (*n* = 6). C. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 10 µM CPA, and 50 mM ethanol with 10 µM CPA. D. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in C and a wash. Event frequency under control conditions was 1.3 ± 0.3 Hz (*n* = 9).

3-3-4. The 5-HT_{2C}R agonist, Ro-60-0175, and 5-HT increase GABA release

Recent studies have linked 5-HT_{2C}R activation with modulation of VTA-GABA and DA transmission (Di Giovanni et al., 2000; Di Matteo et al., 2000; Bankson and Yamamoto, 2004). Therefore, the role of 5-HT_{2C}Rs in the ethanol enhancement of mIPSC frequency was first investigated by assessing whether activation of 5-HT_{2C}Rs modulates baseline GABA release. Application of the selective 5-HT_{2C}R agonist, Ro-60-0175 (200 nM), significantly enhanced mIPSC frequency (Fig. 4A, B) over a time course that developed relatively slowly (Fig. 4B, C) and continued to increase throughout the 12 min following removal of the drug. This pattern of prolonged drug action was similar to the prolonged actions of ethanol described previously. Finally, no change in mIPSC amplitude was observed (data not shown) in the presence of Ro-60-0175 thereby suggesting that 5HT_{2C}R activation solely enhances GABA release. We verified the selectively of the actions of the 5-HT_{2C} agonist by pretreating slices with the 5-$HT_{2B/2C}$ antagonist, SB200646 (4 µM) and under these conditions, the Ro-60-0175-induced increase in mIPSC frequency was completely abolished (Fig. 4D, E; note the different scales used in 4B vs 4E). Additionally, application of 5-HT (50 µM) produced a significant enhancement in mIPSC frequency (Fig. 5) with no subsequent effect on amplitude (data not shown). Similar to the effects of Ro-60-0175, the enhancement by 5-HT did not fully wash out and actually increased beyond wash in 3 of 7 neurons.

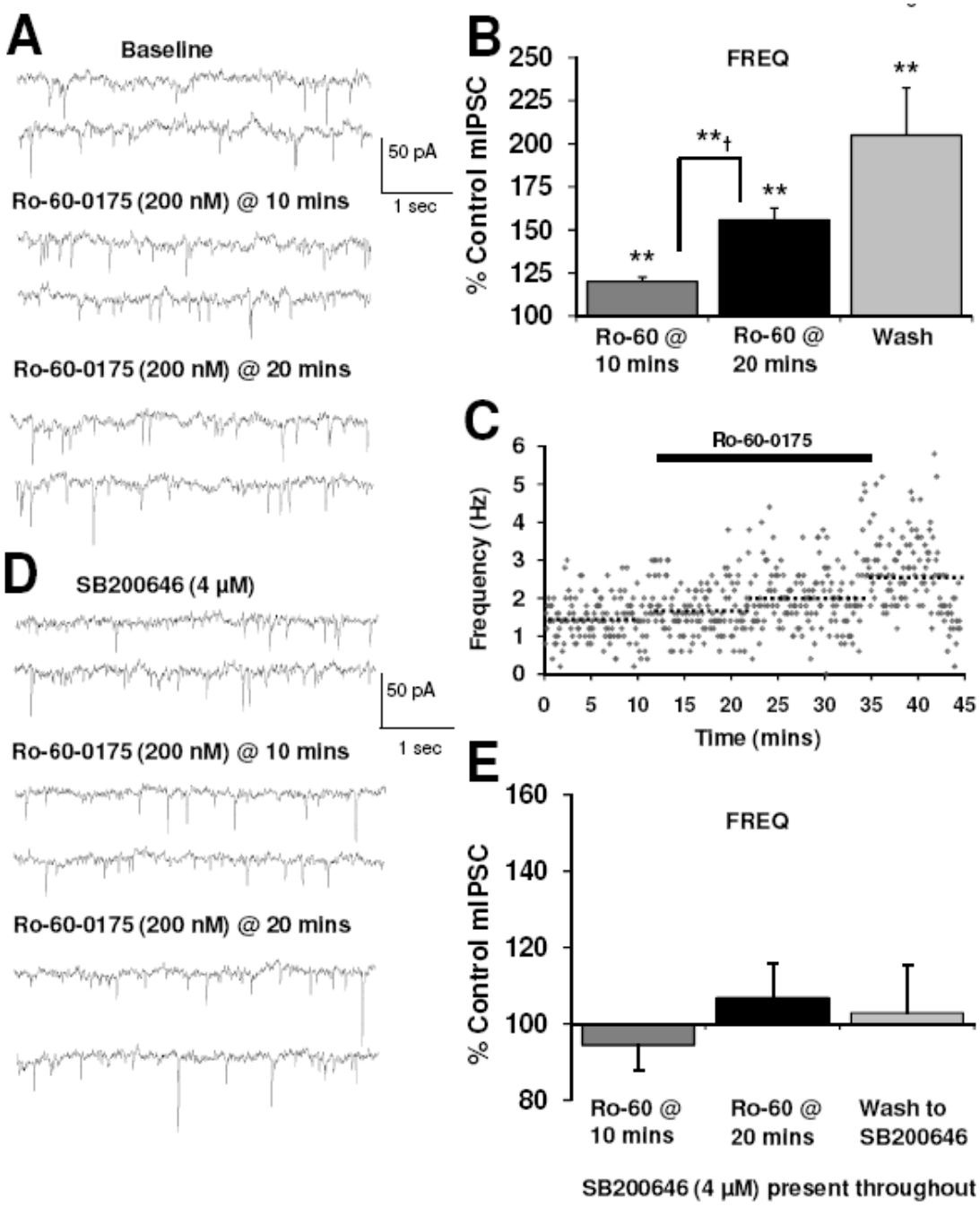

A
Baseline
50 pA
1 sec
Ro-60-0175 (200 nM) @ 10 mins
Ro-60-0175 (200 nM) @ 20 mins
B
FREQ
% Control mIPSC
250
225
200
175
150
125
100
**
**†
**
**
Ro-60 @ 10 mins
Ro-60 @ 20 mins
Wash
C
Ro-60-0175
Frequency (Hz)
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
40
45
Time (mins)
D
SB200646 (4 µM)
50 pA
1 sec
Ro-60-0175 (200 nM) @ 10 mins
Ro-60-0175 (200 nM) @ 20 mins
E
FREQ
% Control mIPSC
160
140
120
100
80
Ro-60 @ 10 mins
Ro-60 @ 20 mins
Wash to SB200646
SB200646 (4 µM) present throughout

Figure 3-4. 5-HT_{2C}R activation increases GABA release onto VTA-DA neurons. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 200 nM Ro-60-0175 (after 10 min), and with 200 nM Ro-60-0175 (after 20 min). B. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A and a wash. Event frequency under control conditions was 1.3 ± 0.2 Hz. (n = 8, ** indicates $p < 0.01$ by student's t test different from control, **† indicates $p < 0.01$ by student's t test different from Ro-60 at 10 min) C. Time course for a sample representative neuron under conditions shown in A. The dotted lines represent the average frequency under each condition for that neuron. D. mIPSCs recorded from a VTA-DA neuron in continued presence of 4 µM SB200646, with subsequent application of 200 nM Ro-60-0175. E. A bar graph representing the % change ± SEM above control mIPSC frequency under for the conditions shown in D and a wash to SB200646. Event frequency under pre-Ro-60-0175 conditions was 1.9 ± 0.7 Hz. (n = 5).

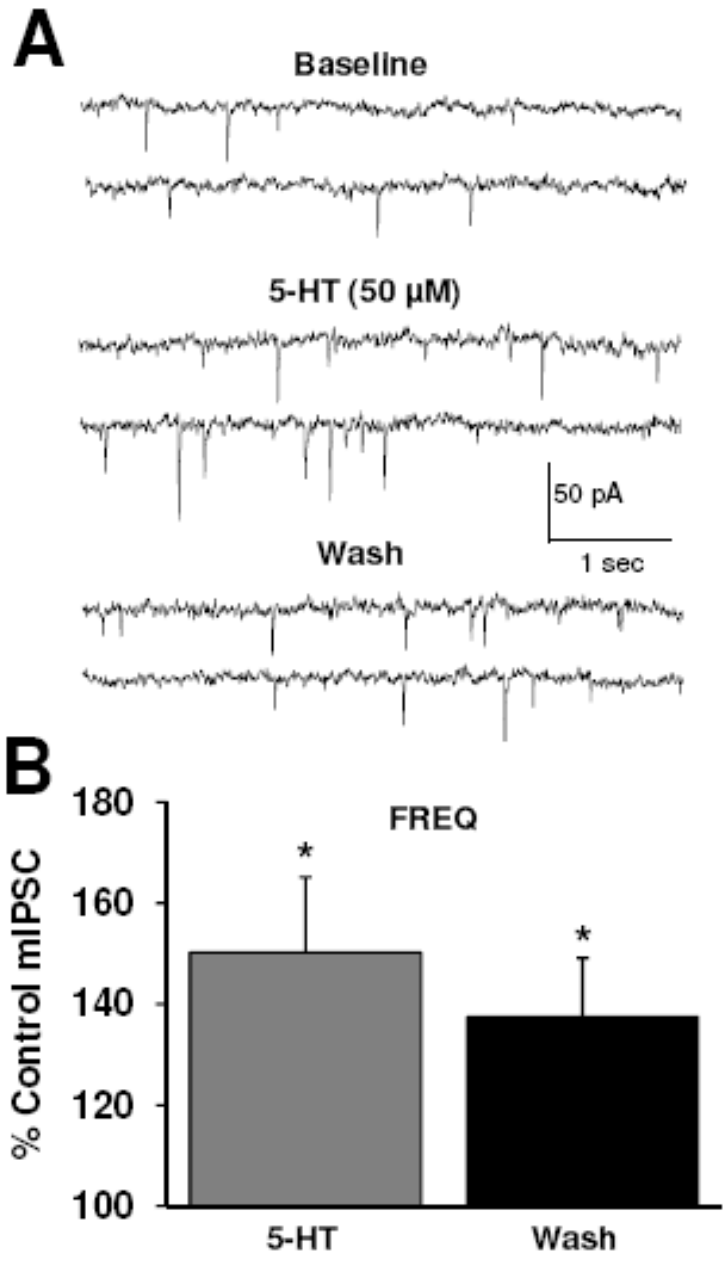


Figure 3-5. 5-HT increases GABA release onto VTA-DA neurons. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 50 µM 5-HT, and after a washout. B. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A. Event frequency under control conditions was 1.2 ± 0.1 Hz. (n = 7, * indicates $p < 0.05$ by a student's t test different from control).

3-3-5. Ethanol-enhancement of GABA release is dependent upon 5-HT_{2C}R stimulated release of Ca^{2+} from intracellular stores

To further validate that mobilization of intracellular Ca^{2+} is required for the 5-HT_{2C} agonist activity, we studied the effect of CPA (10 µM) on Ro-60-0175 enhancement of mIPSC frequency. Addition of the 5-HT_{2C} agonist following bath application of the SERCA pump inhibitor almost completely abolished the agonist-induced enhancement of mIPSC frequency (Fig. 6A, B: 17% vs 56% increase as seen in Fig. 4B). Since ethanol appears to enhance GABA release through an IP_3 cascade which may also be activated by 5-HT_{2C}Rs, then pharmacological blockade of the 5-HT_{2C}R may significantly reduce or abolish ethanol potentiation of mIPSC frequency. Therefore, we pretreated slices with SB200646, for 5-10 minutes prior to application of ethanol (50 mM). SB200646 (4 µM) completely prevented the increase in mIPSC frequency normally observed after ethanol application (Fig. 6C, D). There was no effect on mIPSC amplitude (data not shown). These data suggest that in the VTA, 5-HT_{2C}R activation is necessary for the ethanol-induced increase in GABA release.

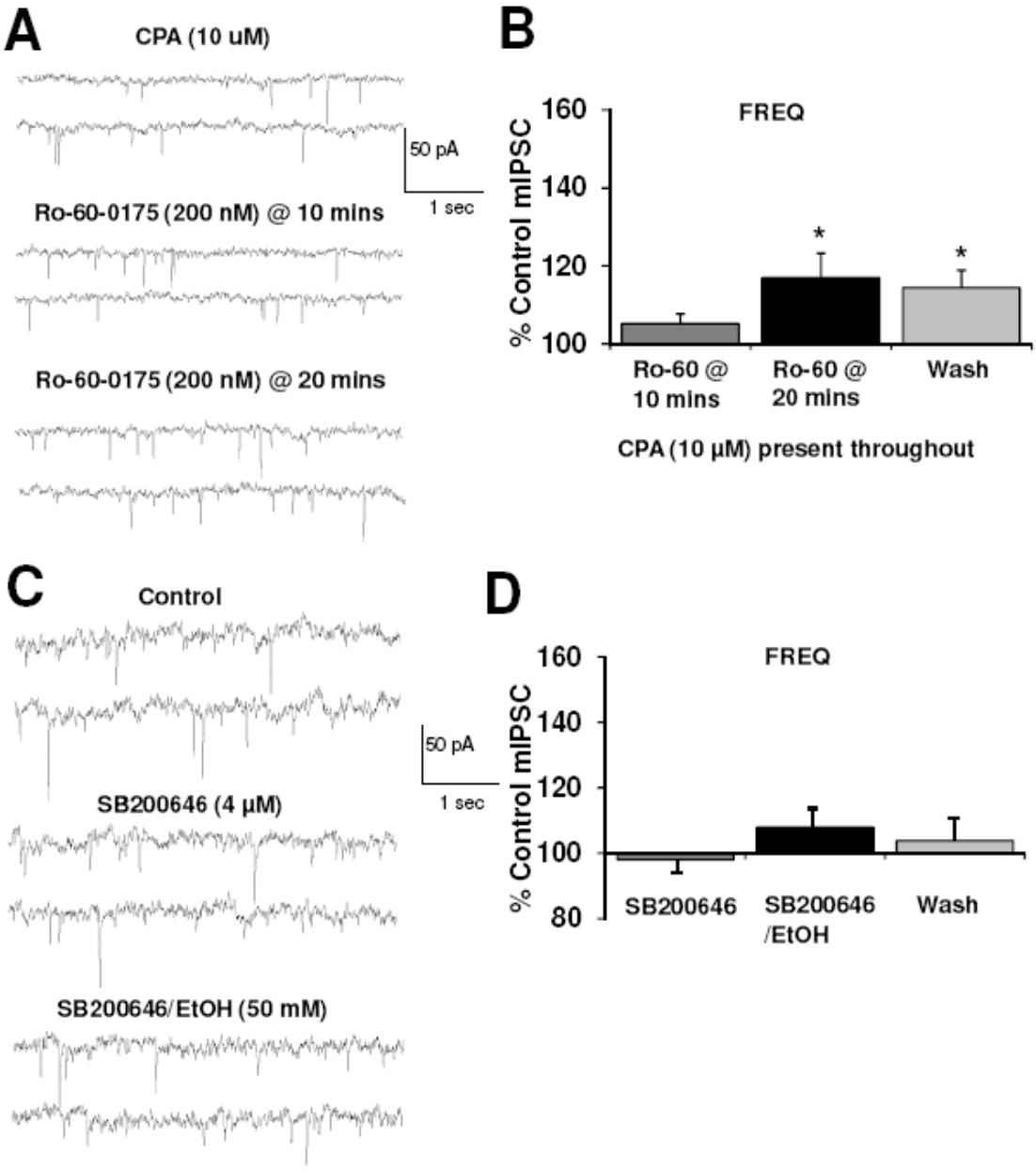

A
CPA (10 uM)
50 pA
1 sec
Ro-60-0175 (200 nM) @ 10 mins
Ro-60-0175 (200 nM) @ 20 mins
B
FREQ
% Control mIPSC
160
140
120
100
*
*
Ro-60 @ 10 mins
Ro-60 @ 20 mins
Wash
CPA (10 µM) present throughout
C
Control
50 pA
1 sec
SB200646 (4 µM)
SB200646/EtOH (50 mM)
D
FREQ
% Control mIPSC
160
140
120
100
80
SB200646
SB200646 /EtOH
Wash

Figure 3-6. Ethanol-enhancement of GABA release is dependent upon 5-HT_{2C}R stimulated release of Ca^{2+} from intracellular stores. A. mIPSCs recorded from a VTA-DA neuron in the continued presence of 10 µM CPA, with subsequent application of 200 nM Ro-60-0175. B. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A. Event frequency under pre-Ro-60-0175 conditions was 1.3 ± 0.2 Hz. (*n* = 6, * indicates $p < 0.05$ by a student's *t* test different from control,). C. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 4 µM SB200646, and 50 mM ethanol with 4 µM SB200646. D. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in C and a wash. Event frequency under control conditions was 2.7 ± 0.4 Hz (*n* = 10).

3-4. Discussion

In our initial report that characterized ethanol-enhancement of VTA-GABA release, we primarily used recordings of spontaneous IPSCs (sIPSCs) which demonstrated that ethanol significantly enhanced sIPSC frequency in a concentration-dependent manner (Theile et al., 2008). Here, we have further investigated a variety of potential mechanisms for the ethanol enhancement through analysis of action potential-independent mIPSCs. Ethanol (15, 25, 50 and 75 mM) clearly increased mIPSC frequency but had no discernable effect on the amplitude, decay of mIPSCs or any tonic GABA current. Baseline mIPSC frequency was reduced in the presence of $CdCl_2$; however neither $CdCl_2$ nor the L-type VGCC antagonist, nicardipine, prevented ethanol enhancement of GABA release. Blockade of IP_3Rs with 2-APB and depletion of intracellular stores with CPA both completely abolished the ethanol-induced increase in mIPSC frequency. Finally, addition of both 5-HT and the 5-HT_{2C}R agonist, Ro-60-0175, increased mIPSC frequency over a similar time course as that seen with ethanol. Blockade with the 5-HT_{2C}R antagonist, SB200646, abolished ethanol enhancement of mISPC frequency as well as that following application of the selective 5-HT_{2C}R agonist. Thus, our primary conclusion is that the ethanol-induced enhancement in GABA release onto VTA-DA neurons is mediated via 5-HT_{2C}R activation which under normal conditions would subsequently release Ca^{2+} from IP_3-sensitive stores.

While our prior report was the first to assess ethanol modulation of GABA release in VTA (Theile et al., 2008), Ye and colleagues recently reported that ethanol (10-40 mM) may have differential effects on VTA action potential-dependent GABA release. Thus, while under control conditions ethanol appeared to decrease GABA release, an increase in sIPSC frequency was observed in the presence of saturating concentrations of the mu-opioid receptor (MOR) agonist DAMGO (Xiao and Ye, 2008). The authors contend that application of DAMGO silences VTA interneurons via activation of somatic and dendritic MORs thereby unmasking the stimulatory effect of ethanol on other GABAergic inputs. To us, the most parsimonious explanation for these somewhat disparate findings is that, under control conditions, our VTA slice preparation must possess a higher level of endogenous opioid tone and thus mimic the conditions demonstrated by Xiao et al. (2008). On the other hand, Bergevin et al. (2002) has reported the presence of MORs on GABAergic terminals in the VTA. This observation complicates the hypothesis presented by Xiao et al. (2008). Nevertheless, it is also important to note that ethanol has been reported to potentiate GABA release in a majority of brain regions studied and therefore our observations in the VTA are consistent with the broad literature (Siggins et al., 2005; Weiner and Valenzuela, 2006).

Activation of VGCCs increases intraterminal Ca^{2+} levels to initiate neurotransmitter release. Therefore, we examined the role that VGCCs may serve in ethanol modulation of GABA release in the VTA. Typically VGCCs, such

as N- and P/Q-type channels, are primarily involved in action potential-dependent neurotransmitter release (Wheeler et al., 1994). Although these experiments were conducted in the presence of TTX to block action potentials, L- and P/Q-type VGCCs have been shown to support GABA release onto Meynert neurons independent of action potentials (Rhee et al., 1999). While application of the non-specific VGCC inhibitor, $CdCl_2$, alone resulted in a decrease in the baseline mIPSC frequency similar to that observed by another group (Bergevin et al., 2002), it did not prevent the stimulatory effect of ethanol. However, the magnitude of ethanol enhancement was slightly reduced compared to the results shown in Figure 1 and this observation may be attributable to a partial depletion of the store as a result of extended blockade of extracellular Ca^{2+} influx. Application of nicardipine, an L-type channel inhibitor, had no effect on baseline mIPSC frequency and similarly did not block the ethanol-induced enhancement in mIPSC frequency. These results suggest that while certain VGCCs contribute partly to basal VTA-GABA release, they are not important in the mechanism of ethanol-enhancement of GABA release.

Since influx of Ca^{2+} through VGCCs does not appear to underlie ethanol stimulation of GABA release, we next investigated the dependency of ethanol enhancement on intracellular Ca^{2+} stores. Ethanol enhancement of intracellular Ca^{2+} levels is well documented in the literature. Early work showed that pharmacologically relevant concentrations of ethanol facilitated Ca^{2+} release from IP_3 sensitive stores in brain microsomes (Daniell and Harris, 1989). Additionally,

release of Ca^{2+} from presynaptic cytosolic pools can facilitate GABA release in a variety of brain regions (Savic and Sciancalepore, 1998; Rhee et al., 1999; Kelm et al., 2007). Application of the IP_3R antagonist, 2-APB, prevented the ethanol enhancement of mIPSC frequency. These findings are comparable to recent observations in the cerebellum (Kelm et al., 2007). High concentrations of 2-APB have been suggested to lack selectivity, yet such issues arise at concentrations higher than 80 µM (Missiaen et al., 2001). Therefore, we used a considerably lower concentration and did not observe any non-specific effects as demonstrated by the lack of change in mIPSC frequency in the presence of 2-APB alone. We further tested the dependency of intracellular Ca^{2+} stores by depleting these stores using the SERCA pump inhibitor, CPA. Application of CPA likewise had no effect on baseline mIPSC frequency and subsequent application of ethanol failed to elicit the reliable increase in mIPSC frequency. Together with the 2-APB results, we conclude that IP_3-mediated Ca^{2+} release is necessary for ethanol-induced enhancement in GABA release.

DA output from the VTA is modulated by 5-HT-containing neurons originating from the midbrain raphe nuclei which innervate both DA and non-DA neurons (Herve et al., 1987). 5-$HT_{2C}R$ activation leads to phosphatidylinositol turnover and subsequent Ca^{2+} release via IP_3R activation (Conn et al., 1986). Modulation of midbrain DA neuron activity via 5-$HT_{2C}Rs$ is believed to occur indirectly through changes in GABAergic transmission (Di Giovanni et al., 2000). Here, we demonstrated a 5-$HT_{2C}R$-dependent increase in GABA release since

application of 5-HT or the selective 5-HT_{2C}R agonist, Ro-60-0175, resulted in a prolonged enhancement in mIPSC frequency of which the latter effect was abolished in the presence of the 5-HT_{2C} selective antagonist, SB200646. This enhancement was also substantially reduced by the SERCA pump inhibitor, CPA, suggesting this component is Ca^{2+} mediated. Most importantly, we observed that blockade with SB200646 abolished the ethanol-mediated enhancement in GABA release. These results are consistent with previous studies. Methylenedioxymethamphetamine ('ecstasy') increases GABA release in the VTA through a 5-HT_{2C}R-dependent mechanism (Bankson and Yamamoto, 2004). In the substantia nigra, local and systemic administration of Ro-60-0175 was observed to stimulate GABA release (Invernizzi et al., 2007). Thus, while we cannot rule out other metabotropic receptors which may also activate GABA release in an ethanol-responsive manner as we report here, it does appear that the 5-HT_{2C}R is a prime candidate for the physiological regulation of GABA release in the VTA and a target of ethanol modulation as well.

Multiple labs have demonstrated that ethanol produces an acute enhancement of VTA-DA neuron excitability. Here and in a previous report (Theile et al., 2008), we observe a seemingly contradictory enhancement in inhibitory drive onto VTA-DA neurons. Thus, ethanol possesses an apparent biphasic action to directly enhance both DA neuron excitability and inhibitory drive onto DA neurons. This concept may have implications for the development of novel therapies for treating alcohol craving. For instance, the MOR antagonist,

naltrexone, is one of the few approved agents for craving. One prominent action of MOR activation is a reduction in GABA release onto VTA-DA neurons, thus enhancing their excitability (Johnson and North, 1992a; Bergevin et al., 2002). Therefore, we hypothesize that a derangement in VTA-GABA function, at least in part, may underlie ethanol dependence and craving. Consequently, one possible therapeutic mechanism of MOR antagonism may be to normalize GABA release and thus amplify the ethanol-induced increase in GABA release to diminish or temper the direct reinforcing action of ethanol on VTA-DA neurons. Since we show herein that activation of 5-HT_{2C}Rs enhances GABA release and is critical in ethanol modulation of GABAergic inhibition onto VTA-DA neurons, novel pharmacotherapies for alcohol craving might focus on this mechanism for directly enhancing inhibitory drive onto these neurons.

Here, we demonstrate that ethanol enhances VTA-GABA release in a manner that is dependent on activation of 5-HT_{2C}Rs, which activate the IP_3 receptor cascade and, subsequently, Ca^{2+} release from intracellular stores. We consider that the most logical site of ethanol action is upstream of the 5-HT_{2C}R. Ethanol increases 5-HT release in the NAc (Yoshimoto et al., 1992) and prevents 5-HT reuptake in the hippocampus (Daws et al., 2006). Therefore, it is conceivable that ethanol may also stimulate 5-HT release in the VTA which subsequently results in the activation of 5-HT_{2C}Rs. This is certainly a possibility given that application of 5-HT enhances GABA release in our preparation. Nevertheless, given the results presented here, 5-HT_{2C}/IP_3R activation

represents a primary candidate for the mechanism by which ethanol enhances GABA release in the VTA and taken together this novel action of ethanol may present new avenues for development of agents to treat alcohol craving.

Chapter 4: Role of GABA and opioids in the regulation of VTA-DA neuron firing rate

4-1. Introduction

$GABA_A$ receptors are present on VTA-DA neurons and GABA release tonically inhibits these neurons (Yim and Mogenson, 1980; Johnson and North, 1992a; Johnson and North, 1992b; Westerink et al., 1996). It is well established that acute ethanol stimulates VTA-DA neuron activity in Sprague-Dawley rats both *in vivo* (Gessa et al., 1985) and *in vitro* (Brodie et al., 1990), as well as in mice (Brodie and Appel, 2000). However, we have evidence demonstrating an ethanol enhancement of GABA release onto VTA-DA neurons (Theile et al., 2008; Theile et al., 2009) implying that ethanol potentially has a dual action on VTA-DA neuron activity. As a result, we hypothesized that the ethanol-enhancement in GABA release may limit the overall stimulatory effect of ethanol on VTA-DA neuron activity.

Furthermore, it has been demonstrated that activation of MORs on GABAergic interneurons in the VTA disinhibits VTA-DA neuron activity (Johnson and North, 1992a; Margolis et al., 2003; Xiao et al., 2007). The MOR agonist, DAMGO, reduced the frequency of miniature and spontaneous IPSCs through inhibition of the secretory process at the nerve terminal of GABAergic cells (Bergevin et al., 2002). In the CeA, ethanol increased GABA release and this effect was enhanced in DOR knock-out mice and in the presence of DOR antagonists (Kang-Park et al., 2007). The authors contend that endogenous

opiate release negatively modulates spontaneous and ethanol-induced GABA release in the CeA. Based on these studies and on our own data showing an ethanol-induced enhancement of GABA release, we hypothesized that the ethanol-induced increase in GABA release is masked by the endogenous opioid tone acting on MORs localized on interneurons. Inhibition of MORs with the non-selective opioid antagonist, naltrexone, may uncover a tonic inhibition of GABA release, thus amplifying the ethanol-induced increase in GABA release. The resultant increase in GABA transmission may then overcome the direct effect of ethanol on DA neuron excitability, thereby providing a possible mechanism to explain the ability of naltrexone to block ethanol-induced increases in dialysate dopamine levels in the NAc of male Wistar rats (Gonzales and Weiss, 1998), and possibly may contribute to the clinical efficacy of naltrexone on preventing relapse in recovering alcoholics.

4-2. Materials and Methods

4-2-1. Slice preparation

All experiments were carried out in accordance with NIH animal use guidelines and were approved by the University of Texas Institutional Animal Care and Use Committee. Slices used in this study were prepared from male Long Evans rats (postnatal day 21 to 28). Rats were anesthetized with halothane, decapitated, and the brain was rapidly removed and placed in an ice-cold choline-based, oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 110 choline Cl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 25 dextrose, 7

$MgSO_4$, 0.5 $CaCl_2$, 11.6 Na-ascorbate and 3.1 Na-pyruvate, bubbled with 95% O_2/5% CO_2 (all chemicals obtained from Sigma-Aldrich, St Louis, MO). Horizontal midbrain slices (210 µm) were prepared using a vibrating slicer (VT1000S; Leica, Nussloch, Germany). The slices were then maintained at 32^oC before electrophysiological recordings for a minimum of 60 minutes in aCSF containing (in mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KCl, 1.23 NaH_2PO_4, 10 dextrose, 2.4 $MgSO_4$, and 1.8 $CaCl_2$, bubbled with 95%O_2/5%CO_2.

4-2-2. Electrophysiological recordings of VTA-DA neurons

Individual slices were transferred to a recording chamber and perfused with oxygenated aCSF (30-32^oC) at a flow rate of ~2 ml/min. Recording aCSF was as described above except it contained 0.9 mM $MgSO_4$ and 2 mM $CaCl_2$. Cells were visualized using IR-DIC optics on an Olympus BX-50WI microscope (Leeds Instruments, Irving, TX). The VTA was identified as being medial to the MT and rostral to the oculomotor nerve and the ml. The majority of recordings were conducted in the lateral VTA, just medial to the MT. For action potential recordings, putative DA neurons were identified by their characteristic slow (1-5 Hz) pacemaking activity. Additionally, whole-cell access was obtained and putative DA neurons were further identified by the presence of a large hyperpolarization-induced I_h current (>200 pA) that was measured immediately following break-in by application of a 1.5-s hyperpolarizing step from -60 to -110 mV (Johnson and North, 1992b). For mIPSC recordings, DA neurons were identified via the presence of an I_h current before the experiment was carried out.

Recording electrodes were made from thin-walled borosilicate glass (TW 150F-4, WPI, Sarasota, Florida, 1.5-2.5 MΩ) and contained (in mM): 135 KCl, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Tris-GTP, pH 7.3 with KOH (all chemicals obtained from Sigma, St Louis, MO). Data were collected by an Axon Instruments Model 200B amplifier filtered at 1 kHz and digitized at 20 kHz (for mIPSCs) and 50 kHz (for action potentials) with a Digidata interface using pClamp v10.2 (Molecular Devices, Sunnyvale, California).

Firing rate recordings were conducted in the absence of any channel/receptor drugs except where mentioned. Action potential firing rate was measured using tight-seal current clamp recordings because rupture of the cell membrane under traditional whole-cell recordings significantly disrupts the pacemaking activity of DA neurons (Morikawa et al., 2003). Following formation of a tight seal and a stable 10-minute baseline (control), drugs were bath applied through the aCSF perfusion line and a continuous 15-17 minute recording epoch was used to detect changes in firing rate. A 10-minute washout followed drug application.

GABAergic mIPSCs were pharmacologically isolated with kynurenic acid (1 mM) to inhibit AMPA- and NMDA receptor-mediated currents. Tetrodotoxin (TTX; 0.5 µM) and eticlopride (250 nM) were included to block Na^+ currents and D_2 receptor-mediated currents, respectively. Under these conditions, mIPSCs were inward at a holding potential of -60 mV and in an initial set of experiments their identity as GABAergic events was verified by testing for block with picrotoxin

or bicuculline (data not shown). Following break-in and a stable 10-minute baseline (control) recording, drugs were bath-applied through the aCSF perfusion line and a continuous 10-15 minute recording epoch was used to detect changes in mIPSC frequency and amplitude. A four-minute drug wash-on preceded the start of data collection in each treatment condition, and a 12-min washout period followed drug application. The number of neurons used per each treatment condition is represented as *n* with only one neuron used per slice. SCH50911, muscimol and DAMGO were obtained from Tocris Bioscience (Ellisville, MO). Kynurenic acid, naltrexone hydrochloride and picrotoxin were obtained from Sigma-Aldrich (St. Louis, MO). TTX was obtained from Alomone Labs (Jerusalem, Israel).

4-2-3. Data analysis

For firing rate recordings, action potentials (30-50 sweeps each condition, 20 sec/sweep) were detected using a threshold detection protocol contained with pClampfit (pClamp v10.2, Axon Inst., Molecular Devices). The peak 5 minutes of the drug effect and the last 5 minutes of washout were normalized to the last 5 minutes of the baseline (control) and represented as a percentage of the control. Averaged values for all data sets are expressed as mean ± SEM and were compared statistically using paired student's *t* test, unpaired student's *t* test and one-way analysis of variance (ANOVA) test where mentioned.

For mIPSC recordings, quantal events (90-120 sweeps each condition, 5 sec/sweep) were detected using the template mIPSC detection protocol

contained within pClampfit. Access resistance ranged from 8 to 25 MΩ and was monitored throughout the experiment. Experiments where access resistance changed (>20% at anytime during the experiment) were excluded from this study. To minimize detecting small noise deflections as mIPSCs (false positives), events <10 pA were discarded. Treatment and washout groups were normalized to the baseline (control) frequency or amplitude and represented as a percentage of the control. Averaged values for all data sets are expressed as mean ± SEM and were compared statistically using paired student's *t* test and unpaired student's *t* test where mentioned.

4-3. Results

4-3-1. Ethanol transiently enhances firing rate of VTA-DA neurons *in vitro*.

Acute ethanol has been demonstrated to increase the firing rate of DA neurons in the VTA of Sprague-Dawley rats both *in vivo* (Gessa et al., 1985) and *in vitro* (Brodie et al., 1990), as well as in mice (Brodie and Appel, 2000). In Long Evans rats, ethanol administration produced a robust transient increase in dopamine release in the NAc as measured via microdialysis (M. Job, unpublished observations). Here, we demonstrate electrophysiologically a similar response in acute brain slices from Long Evans rats. Acute application of ethanol (75 mM) produced a large, transient increase in the firing rate of VTA-DA neurons that reversed upon washout (Fig. 1). This transient increase in firing rate reached a peak of 15.7 $\pm$ 2.1% above baseline within the first five minutes of

ethanol application (termed the ‘rising phase’) before approaching baseline levels even during continued ethanol exposure (termed the ‘falling phase’).

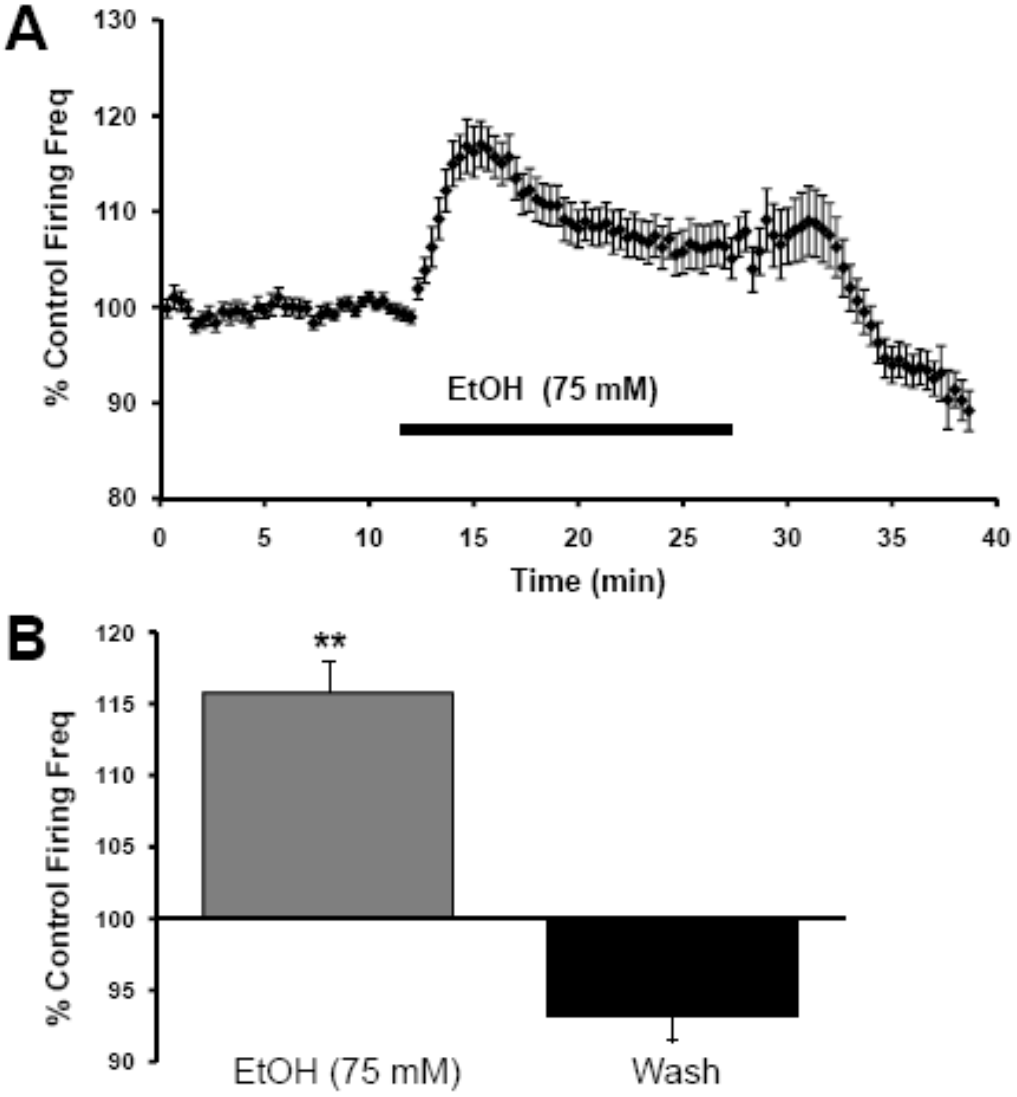


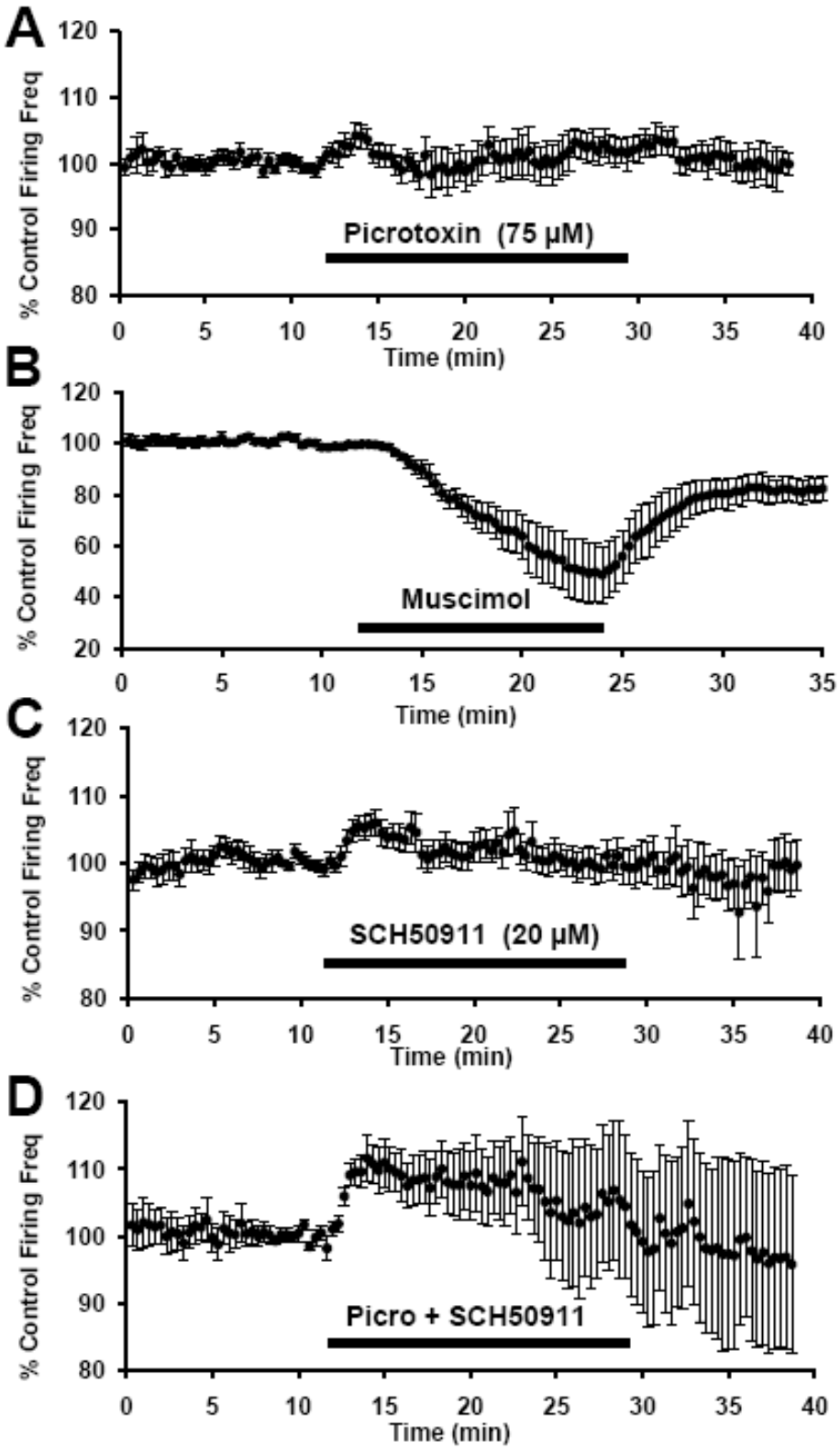
Figure 4-1. Ethanol produces transient enhancement of VTA-DA neuron firing rate. A. Cumulative graph displaying average firing rate of VTA-DA neurons treated with 75 mM ethanol (n = 30). Data points for individual cells represent 20-sec sweeps in which the average firing rate (Hz) was calculated. Each data point is normalized to the last 5 mins of baseline. The normalized data points were combined to obtain the graph above. Error bars represent the SEM of averaged time points. B. A bar graph representing the % change $\pm$ SEM above control firing rate. The bar graph represents the peak 5-min ethanol response and the last 5 mins of washout (n = 30, ** indicates $p < 0.01$ by a student's t test different from control). The average baseline firing rate was 2.3 $\pm$ 0.1 Hz.

4-3-2. GABA modulates VTA-DA neuron firing rate

$GABA_A$ receptors are present on VTA-DA neurons and GABA release has been shown to tonically inhibit these neurons (Yim and Mogenson, 1980; Johnson and North, 1992a; Johnson and North, 1992b; Westerink et al., 1996). Interestingly, some reports suggest the majority of $GABA_A$ receptors are expressed on GABAergic cells and activation of these receptors results in disinhibition, rather than inhibition, of DA neurons in the VTA (Kalivas et al., 1990; Churchill et al., 1992; Xi and Stein, 1998; Laviolette and van der Kooy, 2001; Doherty and Gratton, 2007). We demonstrate here that application of the $GABA_A$ receptor antagonist picrotoxin (75 μM) alone had a disinhibitory effect to increase VTA-DA firing rate (Fig. 2A). Picrotoxin increased the firing rate to a peak of about 4.1 $\pm$ 1.9% above baseline (n = 14, $p<0.05$ by student's t-test). Although the effect is slight, these results give support to the role of postsynaptic $GABA_A$ receptors in modulating DA neuron activity. Conversely, application of the potent $GABA_A$ receptor agonist muscimol (1 μM) substantially inhibited the firing of VTA-DA neurons (Fig. 2B). Muscimol decreased firing rate to about 56.4 $\pm$ 10.4% of baseline (n = 8, $p<0.05$ by student's t-test).

$GABA_B$ receptors are located at pre- and postsynaptic sites in the VTA with activation of presynaptic $GABA_B$ receptors resulting in a decrease in GABA release (Melis et al., 2002). However, some argue that the majority of $GABA_B$ receptors are at postsynaptic sites on DA cells (Xi and Stein, 1998) and activation of these receptors with baclofen decreases DA output to the NAc

(Westerink et al., 1996; Amantea and Bowery, 2004). Therefore we measured the effect of the $GABA_B$ receptor antagonist, SCH50911 (20 µM), on VTA-DA neuron firing rate. In the presence of SCH0911 alone, firing rate was moderately increased (Fig. 2C). SCH50911 increased the firing rate to a peak of about 4.4 $\pm$ 1.9% above baseline (n = 13, $p<0.05$ by student's t-test). This suggests that there may indeed be some postsynaptic $GABA_B$ receptors regulating VTA-DA neuron activity. Lastly, complete blockade of both $GABA_A$ and $GABA_B$ receptors with dual application of SCH50911 and picrotoxin produced an even greater increase in VTA-DA firing rate than with either alone (Fig. 2D). Co-application of picrotoxin and SCH50911 increased the firing rate to a peak of about 12.1 $\pm$ 3.3% above baseline (n = 12, $p<0.05$ by student's t-test). Overall, these results further support the notion that there is enough GABA tone present *in vitro* to modulate VTA-DA activity, and that basal GABA release tonically inhibits VTA-DA neuron activity via activation of both $GABA_A$ and $GABA_B$ receptors.


A
% Control Firing Freq
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5
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15
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Muscimol
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SCH50911 (20 µM)
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Time (min)
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120
110
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80
Picro + SCH50911
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Time (min)

Figure 4-2. $GABA_A$ receptor activation and blockade modulate VTA-DA neuron activity. Cumulative graph displaying average firing rate of VTA-DA neurons treated with (A) 75 µM picrotoxin (n = 14), (B) 1 µM muscimol (n = 8; note different scale), (C) 20 µM SCH50911 (n = 13) and (D) picrotoxin and SCH50911 (n = 12). Data points for individual cells represent 20-sec sweeps in which the average firing rate (Hz) was calculated. Each data point is normalized to the last 5 mins of baseline. The normalized data points were combined to obtain the graph above. Error bars represent the SEM of averaged time points. The average baseline firing rates were for (A) 2.2 $\pm$ 0.3 Hz, (B) 2.5 $\pm$ 0.3 Hz (C) 2.4 $\pm$ 0.3 Hz and (D) 2.1 $\pm$ 0.2 Hz.

4-3-3. Ethanol-enhancement of VTA-DA neuron firing rate is self-limiting

We have previously demonstrated that acute ethanol application results in a long-lasting increase in GABA release onto VTA-DA neurons in Sprague-Dawley rats (Theile et al., 2008; Theile et al., 2009). As previously mentioned, ethanol produced a transient enhancement in VTA-DA firing rate in acute slices from Long Evans rats. We hypothesized that an ethanol-enhancement in GABA release may play a role in the transient nature of the excitatory effect of ethanol on firing rate. As in Sprague-Dawley rats, acute application of ethanol (75 mM) markedly increased mIPSC frequency while having no effect on amplitude in slices from Long Evans rats (Fig. 3). Therefore, ethanol enhances DA neuron activity while concurrently increasing inhibitory drive onto those same neurons. To investigate these opposite actions and their overall impact on VTA-DA neuron activity, we hypothesized that the ethanol-induced increase in GABA release antagonizes the ethanol-induced increase in VTA-DA firing rate and subsequently facilitates a return towards pre-ethanol activity. To test this, we measured the effects of ethanol on VTA-DA firing rate in the continued presence of a saturating concentration of picrotoxin (75 μM). In the absence of picrotoxin, the peak ethanol-induced increase in VTA-DA firing rate was about 16% above baseline (Fig. 1). In the presence of picrotoxin (Fig. 4A), the peak ethanol-induced increase was 19.3 $\pm$ 2.7% above baseline (n=14, $p<0.01$ by student's t-test). When comparing the peak 5 minute ethanol effect between ethanol alone and in the presence of picrotoxin, this difference was significant (One-way

ANOVA, $p<0.01$, $F = 20.55$). Additionally, in the presence of picrotoxin, the elevated firing rate was sustained for a longer period of time before returning towards pre-ethanol levels. When comparing the last 5 minutes of ethanol exposure between ethanol alone and in the presence of picrotoxin, this difference was also significant (One-way ANOVA, $p<0.01$, $F = 195.79$). These results suggest that one of the factors mediating the falling phase of the ethanol response may be a prolonged ethanol-induced enhancement in GABA release. To further support this notion, co-application of muscimol and ethanol uncovered an inhibitory effect of ethanol on VTA-DA neuron firing rate. In the presence of muscimol (1 μM) alone, VTA-DA neuron firing rate was depressed to about 56% of baseline (Fig. 2B) with only 1 of 8 neurons tested exhibiting complete cessation in firing. However, co-application of muscimol and ethanol (75 mM) depressed firing rate to 36.5 $\pm$ 11.5% of baseline with 5 of 9 neurons tested exhibiting complete cessation of firing (Fig. 4B). When comparing the last 5 minutes exposure between muscimol alone and muscimol with ethanol, this difference was significant (One-way ANOVA, $p<0.01$, $F = 45.26$). Thus, under conditions of adjunctive $GABA_A$ receptor activation ethanol inhibits VTA-DA neuron activity, presumably via an enhancement in GABA release.

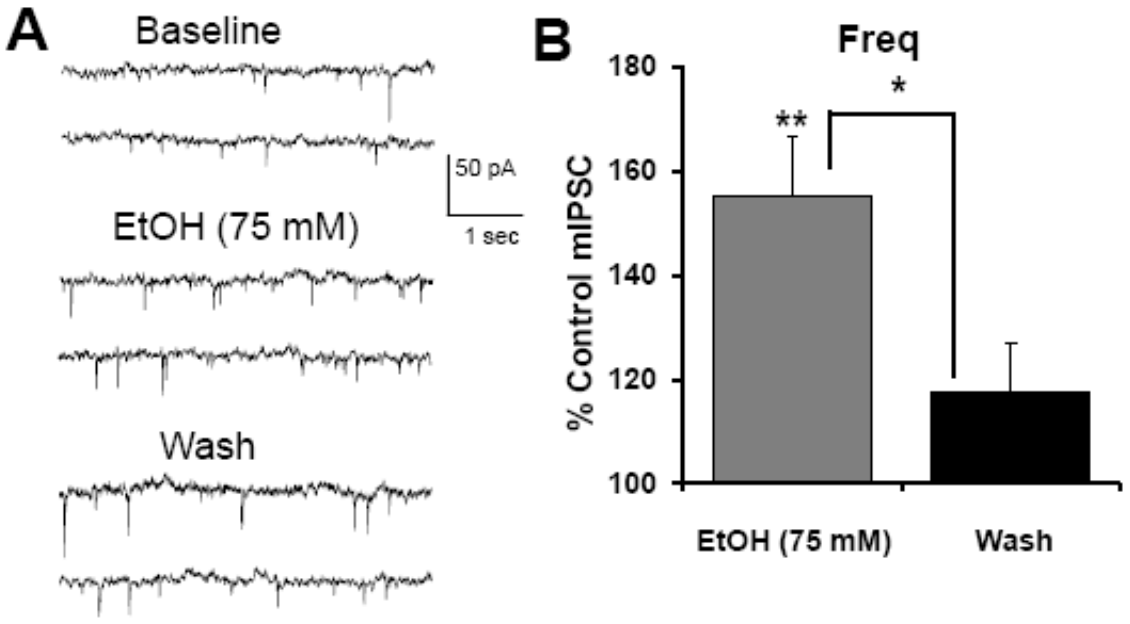

Figure 4-3. Ethanol enhances mIPSC frequency. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 75 mM ethanol and a washout. B. A cumulative bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A. Event frequency under control conditions was 1.1 ± 0.1 Hz (n = 11, ** indicates $p < 0.01$ by a student's t test different from control, * indicates $p < 0.05$ by a student's t test different from EtOH).

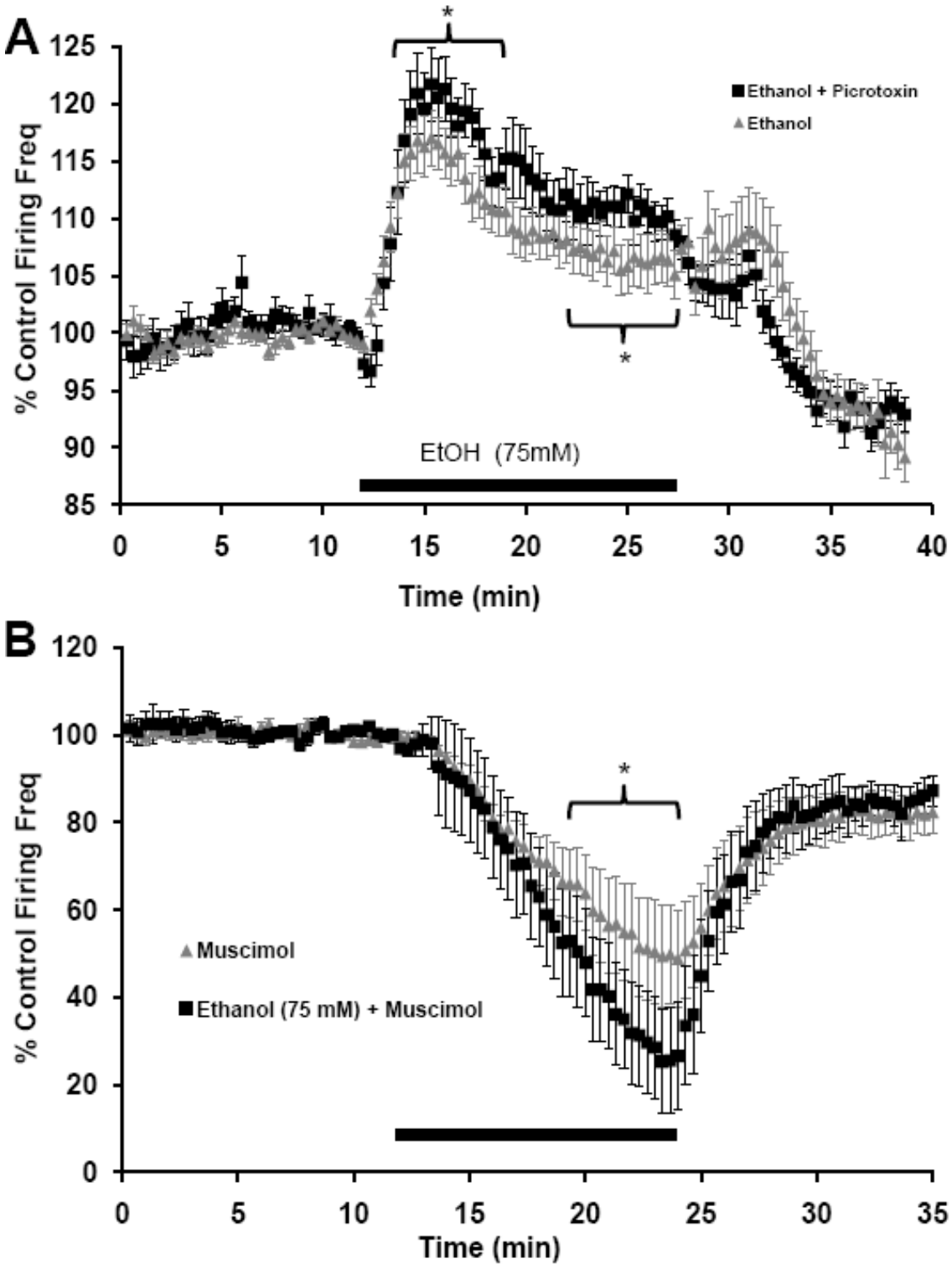
A
% Control Firing Freq
Time (min)
Ethanol + Picrotoxin
Ethanol
EtOH (75mM)
B
% Control Firing Freq
Time (min)
Muscimol
Ethanol (75 mM) + Muscimol

Figure 4-4. Ethanol modulation of VTA-DA activity is regulated by $GABA_A$ receptors. A. Cumulative graph displaying average firing rate of VTA-DA neurons treated with ethanol (75 mM; n = 30; reproduced from Fig. 1) and ethanol (75 mM) in the continued presence of picrotoxin (75 μM; n = 14). The average baseline firing rate for ethanol with picrotoxin was 1.9 $\pm$ 0.1 Hz (* indicates $p<0.01$ by one-way ANOVA). B. Cumulative graph displaying average firing rate of VTA-DA neurons treated with muscimol (1 μM; n = 8; reproduced from Fig. 2B) and ethanol (75 mM) with muscimol (n = 9). The average baseline firing rate for ethanol with muscimol was 2.6 $\pm$ 0.6 Hz (* indicates $p<0.01$ by one-way ANOVA). For both A and B, data points for individual cells represent 20-sec sweeps in which the average firing rate (Hz) was calculated. Each data point is normalized to the last 5 mins of baseline. The normalized data points were combined to obtain the graphs above. Error bars represent the SEM of averaged time points. Note differences in scale.

4-3-4. Mu-opioid receptor activation inhibits GABA release onto VTA-DA neurons

Bergevin et al. (2002) demonstrated a clear effect of DAMGO, a selective MOR agonist, to reduce the frequency, but not amplitude, of both $GABA_A$ receptor-mediated sIPSCs and mIPSCs as recorded from VTA-DA neurons of Sprague-Dawley rats. Additionally, acute ethanol has been demonstrated to enhance opioid levels in the VTA *in vivo* (Jarjour et al., 2009). It is unknown if this occurs in an *in vitro* preparation. Therefore, we investigated whether ethanol application could enhance GABA release in the presence of a MOR agonist. Application of DAMGO alone (3 μM) significantly decreased mIPSC frequency (Fig. 5) while having no significant effect on mIPSC amplitude (data not shown). These results support the idea proposed by Bergevin et al. (2002) that activation of MORs, likely on GABAergic terminals synapsing onto DA neurons, decreases vesicular release at these terminals via an action-potential independent mechanism. Following the decrease in mIPSC frequency by DAMGO alone, co-application of DAMGO (3 μM) and ethanol (75 mM) potentiated mIPSC frequency (Fig. 5). When normalized to DAMGO alone, ethanol enhanced mIPSC frequency by about 32%. Additionally, there is no significant effect on mIPSC amplitude under either condition (data not shown). These results suggest that ethanol acts independently of opioid receptors to enhance GABA release.

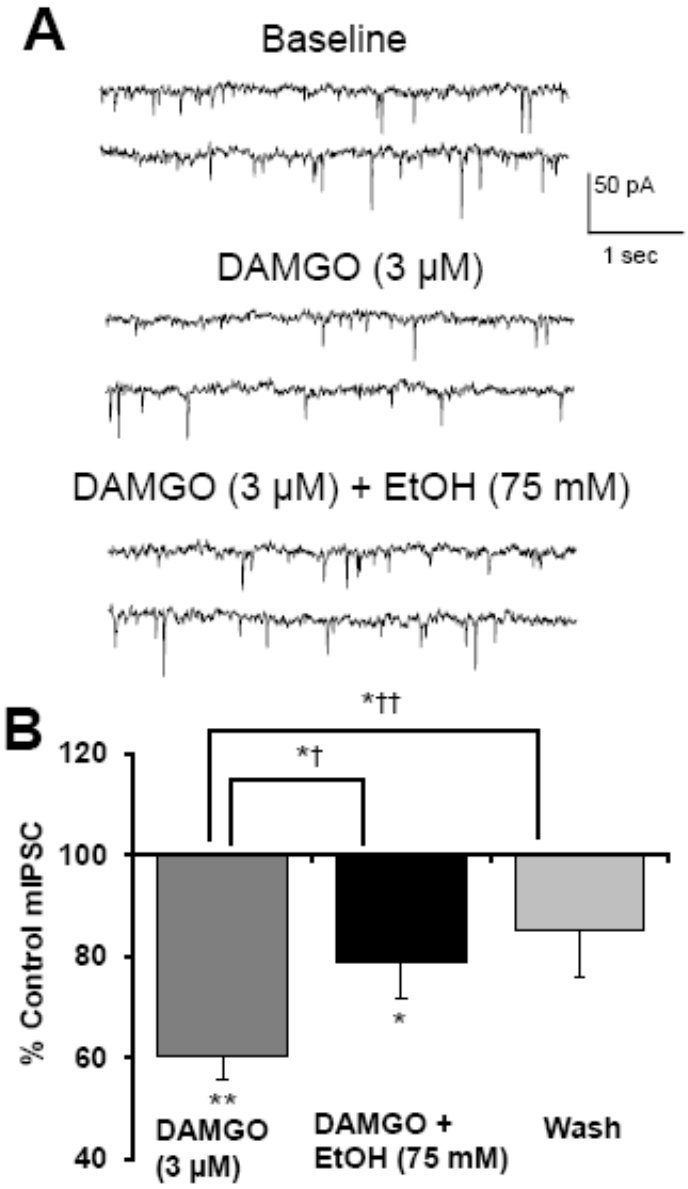

Figure 4-5. DAMGO does not block ethanol-enhancement of mIPSC frequency. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 3 μM DAMGO and 75 mM ethanol with 3 μM DAMGO. B. A cumulative bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A and a wash. Event frequency under control conditions was 1.2 ± 0.2 Hz (n = 8, ** indicates $p < 0.01$ by student's t test different from control, *† indicates $p < 0.05$ by a paired student's t test different from DAMGO, *†† indicates $p < 0.05$ by a paired student's t test different from DAMGO, * indicates $p < 0.05$ by Student's t test different from control).

4-3-5. Mu-opioid receptor activation does not alter VTA-DA neuron firing rate

VTA-DA neuron activity is partly regulated via MOR-mediated inhibition of GABA release. Several labs have demonstrated an MOR-mediated disinhibition of VTA-DA neurons in Sprague-Dawley rats (Johnson and North, 1992a; Margolis et al., 2003; Xiao et al., 2007). Since we observed a substantial decrease in GABA release with DAMGO, we would expect to see a disinhibitory effect of DAMGO on VTA-DA neuron firing rate. Using tight-seal cell-attached current-clamp recordings we measured the firing rate of VTA-DA neurons in the presence of the MOR-selective agonist, DAMGO. The peak response to DAMGO was not significantly greater than the baseline firing rate at 1 µM (n = 8, $p>0.05$ by student's t-test, Fig. 6A) or 3 µM (n = 9, $p>0.05$ by student's t-test, Fig. 6A). We speculate that the presence of a strong GABA tone is necessary in order to see a disinhibitory effect of DAMGO on VTA-DA neuron firing rate *in vitro*. Since there was no change in firing rate at either 1 or 3 µM DAMGO, we raised the extracellular K^+ concentration ($[K^+]_o$) from 3.3 mM to 7 mM. Increasing the $[K^+]_o$ in the recording solution would hypothetically increase GABAergic neuron activity, thus increasing GABA tone in the slice. After establishing a stable baseline firing frequency, the high $[K^+]_o$ solution was washed on before application of DAMGO. Although the addition of the higher $[K^+]_o$ solution dramatically increased the VTA-DA firing rate (73.2 $\pm$ 6.8% above baseline, n = 6, $p<0.01$ by student's t-test), subsequent addition of DAMGO did not result in

any further change in the firing rate (72.9 ± 7.7% above baseline, $p>0.05$ by paired student's t-test compared to high $[K^+]_o$ alone, Fig. 6B). Increasing $[K^+]_o$ not only increases GABA drive onto VTA-DA neurons, but also increases glutamatergic drive as well. Therefore, we repeated the experiment with kynurenic acid in the recording solution to block NMDA- and AMPA receptor-mediated currents (Fig. 6C). Under these conditions, addition of the high $[K^+]_o$ solution increased firing rate to 49.7 ± 11.5% above baseline (n = 7, $p<0.01$ by student's t-test). However, application of DAMGO had no further effect on DA neuron firing rate (38.1 ± 14.4% above baseline, $p>0.05$ by paired student's t-test compared to high $[K^+]_o$ alone). The extent of the increase in firing rate with high $[K^+]_o$ solution in the presence of kynurenic acid did not reach the level as that seen in high $[K^+]_o$ alone. These observations suggest that the high $[K^+]_o$ solution increases glutamatergic transmission while also directly depolarizing VTA-DA neurons. Nevertheless, under no conditions did we observe any evidence of MOR regulation of VTA-DA firing rate.

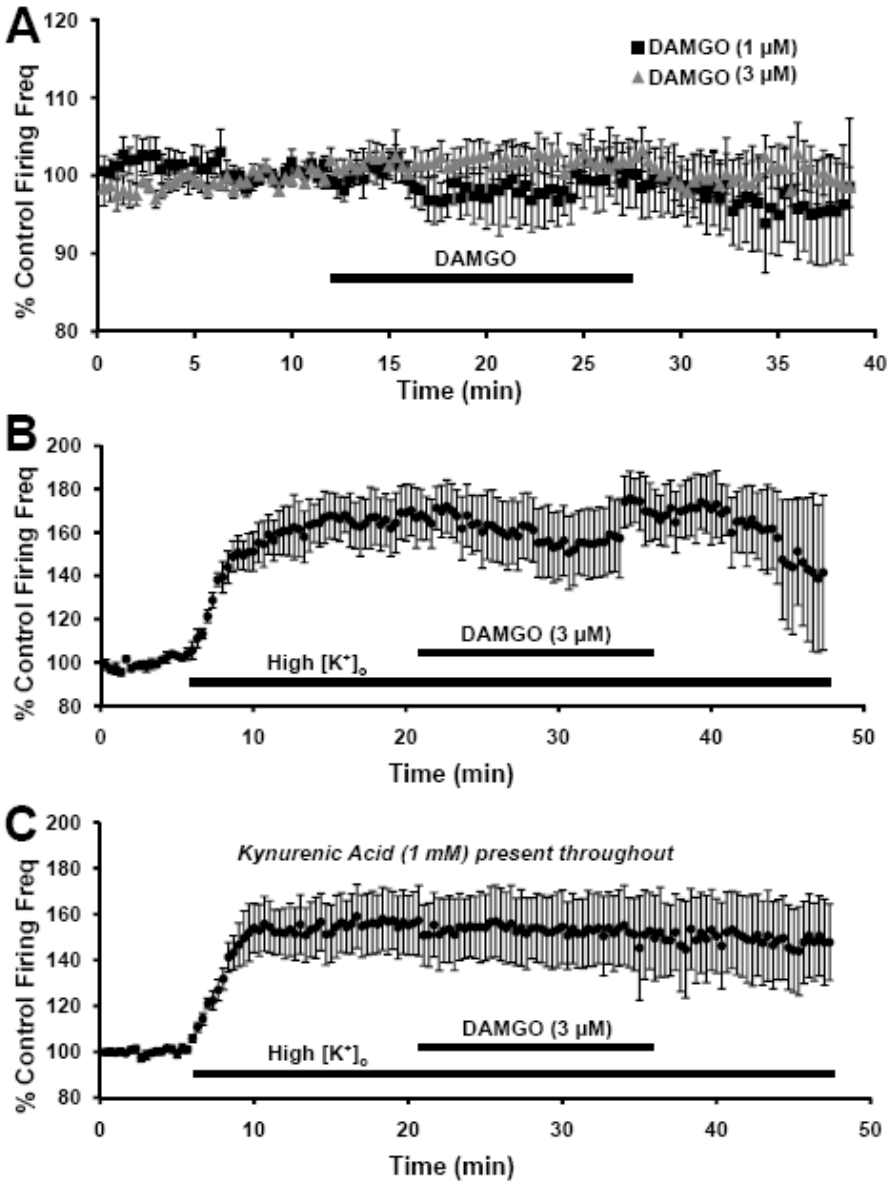

Figure 4-6. DAMGO does not modulate VTA-DA neuron activity. Cumulative graph displaying average firing rate of VTA-DA neurons treated with (A) 1 µM DAMGO (n = 8) and 3 µM DAMGO (n = 9), (B) 3 µM DAMGO with 7 mM $[K^+]_o$ (n = 6) and (C) 3 µM DAMGO with 7 mM $[K^+]_o$ and 1 mM kynurenic acid (n = 7). Data points for individual cells represent 20-sec sweeps in which the average firing rate (Hz) was calculated. Each data point is normalized to the last 5 mins of baseline. The normalized data points were combined to obtain the graph above. Error bars represent the SEM of averaged time points. The average baseline firing rates were for (A) 2.3 $\pm$ 0.2 Hz for 1 µM and 2.7 $\pm$ 0.3 Hz for 3 µM, (B) 1.7 $\pm$ 0.3 Hz and (C) 2.1 $\pm$ 0.3 Hz.

4-3-6. Mu-opioid receptor blockade has no effect on GABA tone or ethanol-enhancement of VTA-DA neuron activity

Blockade of MORs with naltrexone (Gonzales and Weiss, 1998) and the more specific MOR antagonist naloxonazine, along with genetic deletion of MORs, (Job et al., 2007) attenuates ethanol-enhancement of DA release into the NAc, therefore suggesting the opioidergic system positively modulates the excitatory effect of ethanol on DA neuron activity *in vivo*. Furthermore, ethanol increases GABA release in the CeA and this effect is enhanced in DOR knock-out mice and in the presence of DOR antagonists (Kang-Park et al., 2007). The latter study suggests that endogenous opiate release negatively modulates spontaneous and ethanol-enhanced GABA release in the CeA *in vitro*. Together with our own data showing an ethanol-induced enhancement of GABA release in the VTA, we hypothesized that the ethanol-induced increase in GABA release is limited by endogenous or ethanol-enhanced opioid tone acting on MORs localized to interneurons, thus inhibition of MORs with naltrexone may uncover a tonic inhibition of GABA release. The resulting increase in GABAergic transmission in the presence of ethanol may then suppress or inhibit the excitatory effect of ethanol on DA neuron activity. This may provide a possible mechanism to explain the ability of naltrexone to block ethanol-induced increases in dialysate dopamine levels in the NAc. To test this hypothesis, we first examined the effect of naltrexone (60 μM) alone and with ethanol (75 mM) on mIPSCs (Fig. 7). Naltrexone had no effect on mIPSC frequency. Since we did

not observe an enhancement in mIPSC frequency following MOR blockade, this suggests that there is no basal opioid tone in our slice preparation. Naltrexone also did not potentiate the ethanol-enhancement in mIPSC frequency, but in fact the ethanol effect appeared slightly less robust, since when normalized to naltrexone alone, ethanol enhances mIPSC frequency by about 28%.

Additionally, we measured VTA-DA firing rate under normal conditions and in the presence of ethanol (75 mM). Application of naltrexone alone at 20 µM (2.1 + 1.5% above baseline, n = 9, $p>0.05$ by student's t-test) and 60 µM (1.6 $\pm$ 3.1% of baseline, n = 8, $p>0.05$ by student's t-test) had no effect on VTA-DA firing rate. Additionally, naltrexone did not block the ethanol-induced enhancement in VTA-DA firing rate (Fig. 8). In the continued presence of 20 µM naltrexone, ethanol (75 mM) enhanced firing rate by 13.7 $\pm$ 2.2% above baseline (n = 8, $p<0.01$ by student's t-test). In the presence of 60 µM naltrexone, ethanol enhanced firing rate by 21.2 + 3.2% above baseline (n = 11, $p<0.01$ by student's t-test). These results suggest that MOR blockade with naltrexone does not have the same effect on VTA-DA neuron activity *in vitro* as observed *in vivo*.

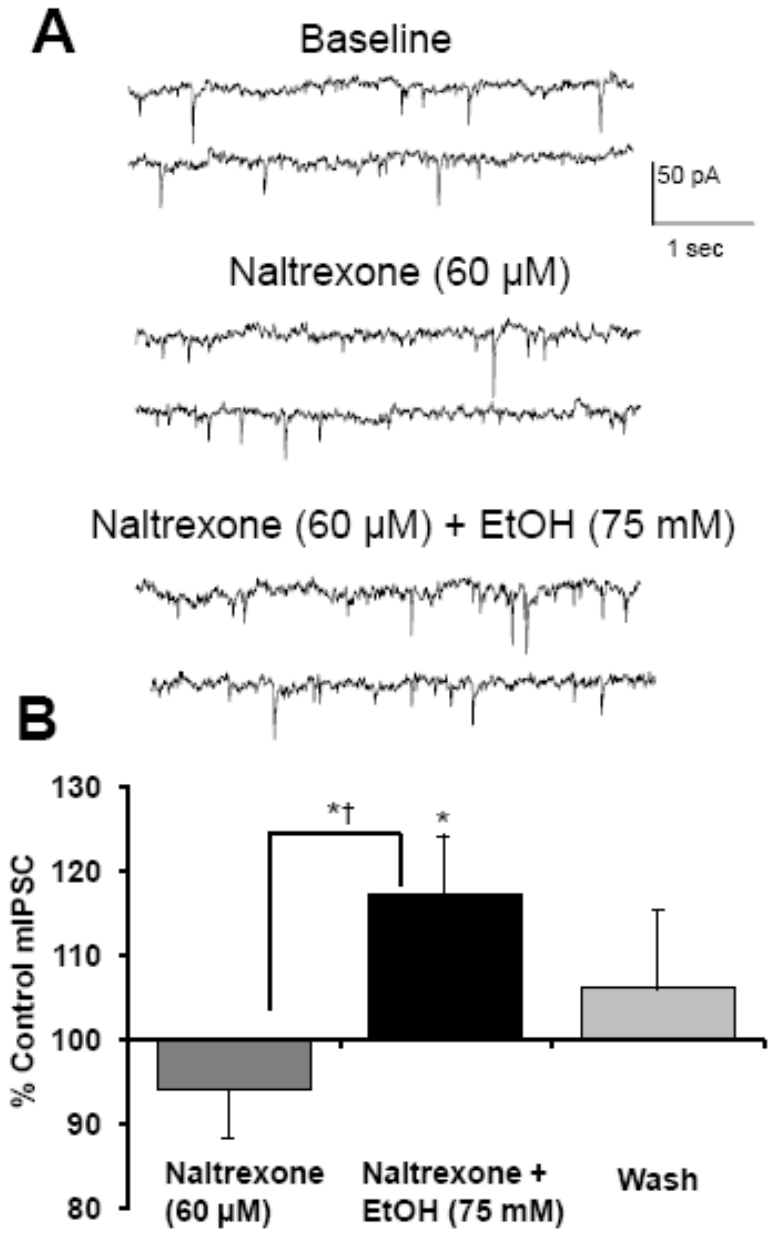

Figure 4-7. Naltrexone does not potentiate ethanol-enhancement of mIPSC frequency. A. mIPSCs recorded from a VTA-DA neuron under control conditions, in the presence of 60 µM naltrexne and 75 mM ethanol with 60 µM naltrexone. B. A bar graph representing the % change ± SEM above control mIPSC frequency for the conditions shown in A and a wash. Event frequency under control conditions was 1.6 ± 0.3 Hz (n = 8, * indicates $p < 0.05$ by student's t test different from control, *† indicates $p < 0.05$ by a paired student's t test different from naltrexone).

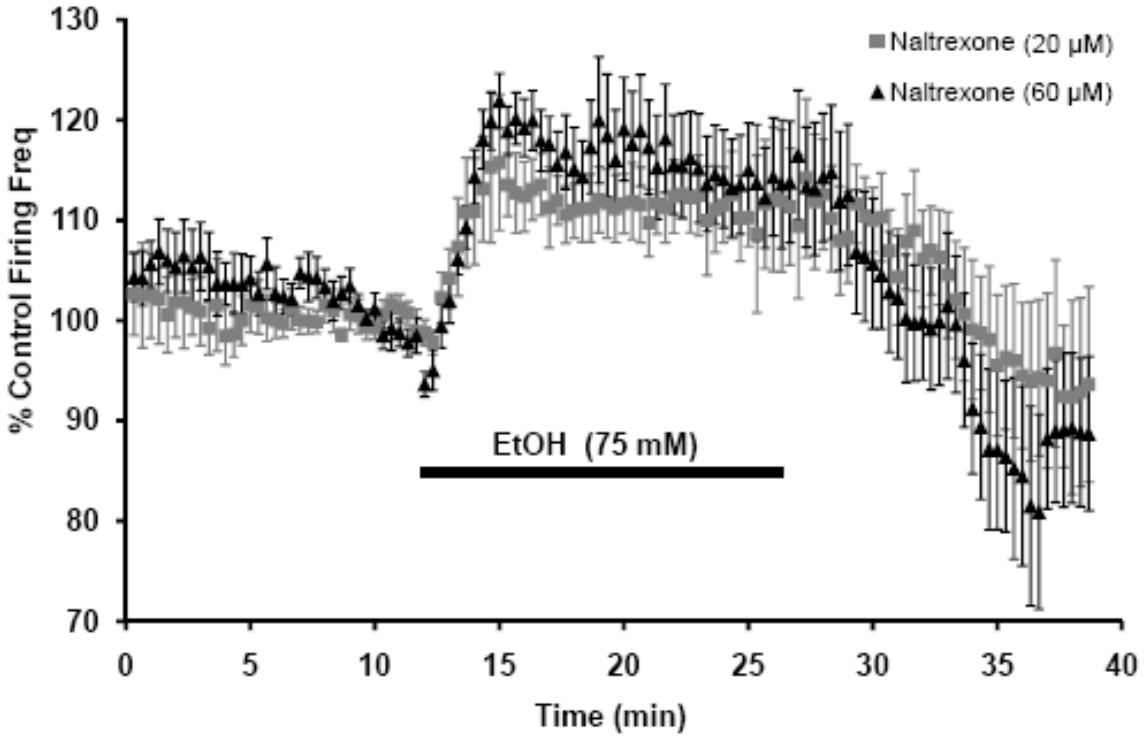

Figure 4-8. Naltrexone does not block ethanol-enhancement in VTA-DA firing rate. Cumulative graph displaying average firing rate of VTA-DA neurons treated with 75 mM ethanol with 20 µM naltrexone present throughout (n = 8) and 75 mM ethanol with 60 µM naltrexone present throughout (n = 11). Data points for individual cells represent 20-sec sweeps in which the average firing rate (Hz) was calculated. Each data point is normalized to the last 5 mins of baseline. The normalized data points were combined to obtain the graph above. Error bars represent the SEM of averaged time points. The average baseline firing rates were for (20 µM naltrexone) 2.4 $\pm$ 0.3 Hz, (60 µM naltrexone) 1.8 $\pm$ 0.1 Hz.

4-4. Discussion

In our initial studies, we demonstrated an ethanol-induced enhancement in GABA release onto VTA-DA neurons of Sprague-Dawley rats (Theile et al., 2008; Theile et al., 2009). From those studies we concluded that the ethanol enhancement in GABA release is likely mediated via 5-HT_{2C} receptor activation and subsequent release of calcium from intracellular stores. Ethanol has been demonstrated to enhance VTA-DA neuron activity *in vivo* (Gessa et al., 1985) and *in vitro* (Brodie et al., 1990); thus in this chapter we have focused on how the ethanol-enhancement of GABA release fits in with the results from those studies and numerous others. Here we demonstrate that acute application of ethanol enhanced both the firing rate of putative VTA-DA neurons as well as GABA release onto those same neurons in Long Evans rats. $GABA_A$ and $GABA_B$ receptor blockade also stimulated VTA-DA neuron activity, thus demonstrating that basal GABA release tonically inhibits VTA-DA neurons in our slice preparation. Moreover, the excitatory effect of ethanol on VTA-DA neuron activity appears to be partly limited by a concurrent ethanol-enhancement in GABA release. In the presence of picrotoxin, ethanol stimulation of VTA-DA firing was significantly more robust and sustained at a greater level, whereas in the presence of muscimol, ethanol inhibited VTA-DA firing. Additionally, MOR activation with DAMGO decreased GABA release but had no apparent disinhibitory effect on VTA-DA neuron firing rate. Conversely, MOR blockade with the antagonist naltrexone had no effect on either basal or ethanol-enhanced

GABA release. Although naltrexone has been demonstrated to block the stimulatory effect of ethanol on VTA-DA neuron activity *in vivo* (Gonzales and Weiss, 1998; Job et al., 2007), we did not observe this result in our slice preparation.

Ethanol (75 mM) application produced a robust and reliable increase in VTA-DA neuron firing rate that consisted of two phases: a rising and falling phase. Immediately after ethanol was introduced into the bath, there was a rapid increase in firing rate that peaked for a few minutes before slowly falling and stabilizing at a level intermediate between the baseline and peak responses. These results suggest that the maximal excitatory effect of ethanol on VTA-DA excitation is not maintained throughout the entire duration of ethanol exposure, but rather reaches an initial peak before some other process acts to diminish the excitation to that approaching pre-ethanol conditions. In this report we demonstrated that GABA modulates VTA-DA firing rate and that ethanol enhances GABA tone. As a result, we hypothesized that an ethanol-induced enhancement in inhibitory drive is one factor that dampens the excitatory effect of ethanol on firing rate. Indeed, in the presence of saturating concentrations of picrotoxin (75 μM), the peak excitatory effect of ethanol was larger and sustained for a longer period of time. Furthermore, while the $GABA_A$ receptor agonist muscimol strongly inhibited VTA-DA firing rate, this inhibition was more pronounced in the presence of ethanol. Thus, while ethanol-enhancement of GABA release alone is not enough to overcome the direct excitatory effect of

ethanol on VTA-DA neuron activity, these results demonstrate that conditions of adjunctive $GABA_A$ receptor activation can reverse the stimulatory effect of ethanol on VTA-DA neuron activity. We speculate that $GABA_A$ receptor activation by muscimol antagonizes the initial excitatory effect of ethanol on firing rate and a long-lasting ethanol-induced enhancement in GABA release produces further inhibition. Thus, these results support the idea that the excitatory effect of ethanol on VTA-DA neuron activity is self-limiting and that $GABA_A$ receptors represent a 'gain switch' regulating the overall stimulatory/inhibitory effect of ethanol.

$GABA_A$ receptors are present on VTA-DA neurons and GABA release tonically inhibits these neurons (Yim and Mogenson, 1980; Johnson and North, 1992a; Johnson and North, 1992b; Westerink et al., 1996). Interestingly, some reports suggested the majority of $GABA_A$ receptors are expressed on GABAergic cells and activation of these receptors results in disinhibition, rather than inhibition, of DA neurons in the VTA (Kalivas et al., 1990; Churchill et al., 1992; Xi and Stein, 1998; Laviolette and van der Kooy, 2001; Doherty and Gratton, 2007). However, here we demonstrate that blockade of $GABA_A$ receptors disinhibited VTA-DA firing and activation of $GABA_A$ receptors strongly inhibited VTA-DA firing. These results suggest that $GABA_A$ receptors are primarily expressed on VTA-DA neurons.

VTA-DA neuron activity is partly regulated via MOR-mediated inhibition of GABA release. Several labs have demonstrated an MOR-mediated disinhibition

of VTA-DA neurons in Sprague-Dawley rats (Johnson and North, 1992a; Margolis et al., 2003; Xiao et al., 2007). It should be noted, however, that in the study by Margolis et al. (2003), disinhibition was seen in only 47% of the neurons tested. Additionally, in the study by Johnson and North (1992a), the use of an elevated $[K^+]_o$ solution was required to observe disinhibition. In line with the results of another study (Bergevin et al., 2002), we demonstrate that DAMGO application decreased mIPSC frequency consistent with an MOR-mediated inhibition of GABA release. Therefore, we investigated whether the decrease in GABA tone would translate to an increase in the excitability of VTA-DA neurons. In the presence of DAMGO (1-3 μM), there was neither a change in DA neuron firing rate under normal recordings conditions nor in the presence of an elevated $[K^+]_o$ solution and kynurenic acid. These results are surprising considering that we did observe a considerable reduction in GABA release in the presence of DAMGO. It is possible that the addition of the higher $[K^+]_o$ solution created a 'ceiling effect' on the VTA-DA neuron activity, thus preventing any further excitation upon addition of DAMGO. Interestingly, kynurenic acid appeared to dampen the overall excitability of the neurons in the presence of the higher $[K^+]_o$ solution. This suggests that glutamatergic terminals are also excited with the higher $[K^+]_o$ solution. In the study by Xiao et al. (2007), they observed a disinhibitory effect of DAMGO on DA neuron excitability in the VTA of Sprague-Dawley rats under normal recording conditions. We cannot fully explain why we do not see similar results; however, the use of coronal slices in that study may

partly explain the different observations seen here. We also demonstrate that ethanol application enhanced GABA release even in the presence of DAMGO. This is important considering that acute ethanol has been demonstrated to enhance endogenous opioid levels in the VTA *in vivo* (Jarjour et al., 2009) and MOR activation inhibits GABA release. Thus, as suggested previously (Theile et al., 2009), the mechanism of ethanol modulation of GABA release is clearly distinct from that of opioid modulation.

Naltrexone has been demonstrated to reduce the ethanol-induced enhancement in dopamine release from the VTA (Gonzales and Weiss, 1998; Job et al., 2007). However, the exact mechanism for this is unclear. We demonstrate here that ethanol reliably enhanced GABA release in the VTA and that GABA release is modulated via MOR activation. Therefore, we hypothesized that if there is opioid tone in our slice preparation, then blockade with naltrexone may enhance GABA tone resulting in a greater ethanol effect on GABA release. Indeed, genetic deletion of MORs in the CeA of mice increased GABA tone (Kang-Park et al., 2009). Upregulation of GABA tone could overcome a direct excitatory effect of ethanol on DA neuron activity. However, we do not observe any change in GABA release in the presence of naltrexone (20-60 μM). Moreover, naltrexone did not block the ethanol-induced enhancement in VTA-DA neuron firing rate. Thus, we do not observe *in vitro* the ability of naltrexone to suppress the ethanol enhancement in VTA-DA neuron activity as seen *in vivo*. Opioid tone is likely substantially reduced or absent in

our slice preparation due to deafferentation, thus representing one of the few drawbacks to studying synaptic transmission in an *in vitro* slice preparation. Similarly, DOR antagonism in the VTA has no effect on GABA release although application of a DOR agonist strongly suppresses GABA release in low-drinking animals (Margolis et al., 2008). The results of that study also suggest that opioid tone is absent in a slice preparation. Furthermore, in the study by Kang-Park et al. (2009) there is no observable effect of MOR deletion on ethanol-enhancement of GABA release in the CeA, although they previously demonstrated an ethanol interaction with DORs (Kang-Park et al., 2007). Conversely, in the hippocampus, naltrexone at 60 μM, but not 30 μM, inhibits the ethanol-induced enhancement in sIPSC frequency (Li et al., 2009).

Interestingly, in the presence of naltrexone (60 μM), the ethanol-enhancement in firing rate did not exhibit the pronounced falling phase as seen with ethanol alone. We can only speculate as to the reason for this result. In addition to being localized to cell bodies and axon terminals of GABAergic neurons in the VTA, MORs are also present on a subset of DA cell bodies and dendrites (Garzon and Pickel, 2001). These DA neurons are classified as tertiary neurons and are differentiated from principle neurons in that they are inhibited by MOR agonists (Cameron et al., 1997; Margolis et al., 2003). Therefore, if ethanol application increases opioid release under our conditions, it is possible that MOR blockade would promote a longer lasting ethanol-induced excitation of DA neuron activity. Additionally, KORs are also present on principle DA neurons and

activation of these receptors results in inhibition of firing (Margolis et al., 2003). Naltrexone is non-selective, especially at high concentrations, thus KOR blockade may result in a disinhibitory effect on firing rate and could explain our observations.

Overall, these results highlight the differences in VTA-DA activity when measuring *in vitro* versus in *vivo*. Although we observed an apparent modulatory role of GABA on ethanol stimulation of VTA-DA firing rate, the same cannot be said for opioid modulation. The results presented here do help to reconcile the fact that ethanol stimulates VTA-DA neuron activity while simultaneously enhancing GABAergic input onto those same neurons. For the first time, we observed an inhibitory effect of ethanol on VTA-DA neuron firing under conditions of additional $GABA_A$ receptor activation. This may have implications with regards to the use of $GABA_A$ agonists in the treatment of alcohol dependence. GABA, and perhaps opioids, may not be the only factors mediating the falling phase of the ethanol-enhancement in VTA-DA neuron activity. Ethanol directly excites VTA-DA neurons through a variety of mechanisms including, but not limited to, enhancement of the I_h current (Okamoto et al., 2006) and a reduction in a voltage-dependent K^+ current (Koyama et al., 2007). The falling phase of the ethanol response could be partly attributable to a development of an acute tolerance of one or more of these elements. In conclusion, the results presented here confirm previous findings in other animal models but give new insight into

the mechanism of ethanol modulation of VTA-DA neuron activity while also highlighting the limitations of studying synaptic networks in a slice preparation.

Chapter 5: Concluding remarks and future studies

An important question that remains concerns the relevance of the present findings to the mechanism of alcohol dependence. This question can be better answered by studying the effects of both acute and chronic ethanol on synaptic transmission in the VTA. Therefore, any forthcoming conclusions from the present study must reflect the fact that there have been no studies investigating the effects of chronic ethanol exposure on GABA release in the VTA. However, one study did demonstrate that VTA-DA neurons are sensitized to ethanol after chronic exposure since acute application of ethanol enhanced VTA-DA firing more in chronically treated rats than in ethanol-naïve rats (Brodie, 2002). Notably, the sensitivity of DA neurons to GABA was reduced in chronically treated animals. Whether or not decreased sensitivity to GABA of VTA-DA neurons reflects any compensatory changes due to changes in GABAergic transmission remains uninvestigated.

Obviously, ethanol is used intermittently and our understanding of changes in VTA-DA and GABA interactions during withdrawal is less well-documented. Several studies have examined the effects of long-term ethanol administration on GABAergic transmission; however, there is a lack of consensus between brain regions and in the type of ethanol administration paradigm used. In the hippocampus, withdrawal from chronic intermittent ethanol (CIE) treatment results in a decrease in mIPSC frequency as recorded from CA1 neurons (Cagetti et al., 2003). Although subsequent exposure to acute ethanol was not

examined in that study, a previous study in the hippocampus demonstrated an enhancement in ethanol sensitivity to evoked IPSCs (Kang et al., 1998). Conversely, in the amygdala, continuous chronic ethanol exposure results in an enhancement in GABA release (Roberto et al., 2004). Furthermore, the authors observed that acute ethanol similarly enhanced GABA release in both chronic treated and ethanol naïve rats, thus suggesting a lack of tolerance to acute ethanol. Nonetheless, based on the results presented by Brodie (2002) there must be some factor regulating the altered sensitivity of VTA-DA neurons to $GABA_A$ receptor-mediated inhibition. Prolonged GABA release induced by ethanol may impart compensatory changes in $GABA_A$ receptor expression and/or function to account for the results seen by Brodie (2002). Indeed, chronic GABA release can decrease $GABA_A$ receptor subunit expression and $GABA_A$ receptor-mediated inhibition (Kumar et al., 2009). Furthermore, Melis et al. (2002) demonstrated that a single *in vivo* injection of ethanol resulted in a long-lasting enhancement in GABA release. Thus it is possible that ethanol-induced changes in GABA release could contribute to the altered VTA-DA sensitivity to GABA-mediated inhibition. As a result, it would be pertinent to investigate the effects of long-term ethanol exposure on GABA release in the VTA, *in vitro* and *in vivo*, using multiple ethanol administration paradigms.

When examining the results of the present study we must also ask whether the observed effects of ethanol on GABAergic transmission and subsequent regulation of VTA-DA neuron activity demonstrated *in vitro* occur *in*

vivo. We clearly demonstrated a discrepancy in opioid receptor modulation of basal and ethanol-enhanced VTA-DA neuron activity between our *in vitro* preparation and *in vivo* preparations from other studies (Di Chiara and Imperato, 1988; Klitenick et al., 1992; Yoshida et al., 1993; Gonzales and Weiss, 1998; Mathon et al., 2005; Job et al., 2007). This discrepancy highlights the importance of repeating many of the same experiments described in this study in the whole animal. No studies have investigated ethanol-mediated changes in GABA levels in the VTA *in vivo* and such an investigation would be a logical next step. Additionally, it would be interesting to investigate whether local application of picrotoxin or muscimol to the VTA could modulate the ethanol-induced increase in NAc dopamine levels.

As demonstrated in the results of Chapter 4, $GABA_A$ receptor activation appears to function as a switch regulating VTA-DA activity in the presence of ethanol. $GABA_A$ receptor blockade results in a greater ethanol-mediated enhancement in VTA-DA neuron firing rate as compared to normal conditions. Conversely, $GABA_A$ receptor activation with muscimol uncovers an ethanol-mediated inhibition in VTA-DA neuron firing rate. This switch from ethanol stimulation to inhibition may have implications for the rewarding and reinforcing properties of ethanol. In the whole animal, strong ethanol stimulation of neighboring brain regions with GABAergic projections to the VTA in conjunction with local GABAergic stimulation could overcome the direct excitatory effect of ethanol on VTA-DA neuron activity. Additionally, in those that become

dependent, basal and/or ethanol-enhanced GABA release may initially be minimal or become weaker after chronic exposure. This would correlate to a more robust excitatory effect of ethanol on VTA-DA neuron activity and thus ethanol consumption could potentially be more reinforcing. Conversely, those who are less susceptible to dependence may exhibit robust basal and/or ethanol-enhanced GABA release, thereby curbing the excitatory effect of ethanol on VTA-DA neuron activity. This in turn would theoretically limit the reinforcing attribute of ethanol on the mesolimbic system. It would be very interesting to investigate potential differences in the acute effects of ethanol on GABA release between strains of animals that are genetically pre-disposed to drink more or less. Those animal strains which are genetically prone to drink less or not at all (alcohol non-preferring) should exhibit a strong basal GABA tone and/or a robust ethanol-enhancement in GABA release which would correlate with a weaker reinforcing effect of acute ethanol. Conversely, those strains which are genetically prone to drink more (alcohol preferring) may exhibit a weak GABA tone and/or a weaker ethanol-enhancement in GABA release and thus exhibit a strong reinforcing effect of ethanol. In fact, in Sardinian alcohol-preferring rats, the frequency of mIPSCs recorded from VTA-DA neurons under control conditions was considerably less than that in non-preferring rats (Melis et al., 2009).

In conclusion, this dissertation effectively demonstrates a stimulatory effect of ethanol on GABAergic transmission in the VTA. 5-HT_{2C} receptor

activation and subsequent release of calcium via intracellular stores represents at least one mechanism by which ethanol stimulates GABA release. Interestingly, ethanol appears to have dual actions on VTA activity whereby ethanol excites VTA-DA neurons directly while simultaneously stimulating GABA release onto those neurons. Obviously, to gain a better and potentially more accurate understanding of the effects of ethanol on VTA activity it would be prudent to repeat many of these experiments in the whole animal. Nonetheless, the results presented here represent novel findings that can further our understanding of the acute effects of ethanol in the VTA and could lead to the development of better treatments for alcohol dependence.

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Vita

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